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Novel components of Pseudomonas putida biofilm exopolymeric matrix and a transcriptome analysis of the effects of osmotic and matric stress

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Novel components of *Pseudomonas putida* biofilm exopolymeric matrix and a transcriptome analysis of the effects of osmotic and matric stress

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Table of Contents

Dedicated to Chelsey Elizabeth Spencer and my family that loves us both

General Introduction

Bacteria have the reputation of being 'simple' life forms based on their single cell nature and lack of a nucleus. However, no other domain on earth contains species that are so diverse in their metabolic and physiological capabilities allowing them to persist in environmental conditions inhospitable to many plants and animals. The key ability to persist in these environments lies in how cells are able to sense and respond to changes in their abiotic conditions. Nutrient abundance, pH, temperature, oxygen, redox state, and water availability are six major factors that are essential for all life. In soil habitats the availability of water is of particular importance since these habitats become routinely or periodically dehydrated by drought or drainage events. The availability of water is measured in terms of its water potential (ψ) and is influenced by both the concentration of solutes (solute potential) and the physical sorption of water to surrounding surfaces (matric potential), which increases as habitats dry. The metabolic capabilities of soil microbes can influence global events such as nitrogen and carbon cycling, since metabolic and physiological competence of bacterial cells are influenced by solute and matric potentials. One way to diminish water loss from cells is to form surface adhered aggregates of cells that are enmeshed within exopolymeric substance of their own making, formally known as biofilms. Some materials within in the biofilm exopolymeric matrix, such as exopolysaccharides (EPS), are hygroscopic and absorb water to help maintain cell hydration. Other matrix components may function in biofilm development and stabilization of the biofilm matrix, aiding to long-term preservation of the biofilm lifestyle.

Progress towards understanding the biofilm properties and matrix components utilized during low water potential conditions is necessary for full appreciation of the factors that influence bacterial survival and function within terrestrial environments. Recent advances in whole genome transcriptomics allow us to explore the effects of reductions in solute and matric potential on *Pseudomonas putida* gene expression that was unavailable to us a decade ago. Also, our understanding of biofilm architecture and molecular interactions within the biofilm matrix has surpassed original perceptions that

biofilms are simply a disordered assemblage of cells. Knowing that there are particular functions related to the types and interactions of exopolymeric substances underscores our need to expand our current understanding of the biofilm matrix and its relationship to water stress physiology. To this end, we explored the types of exopolysaccharides and a novel adhesin, purli, produced by *P. putida* mt2 and speculate on their roles in biofilm formation. Microarray analysis of *P. putida* gene expression changes following solute or matric stress shock conditions identified genes that may produce products important for biofilm formation and water stress tolerance. Together, a firm understanding of the complex genetic and physical traits involved in biofilm formation and water stress physiology will equip us to better understanding bacteria and fitness traits they employ during a variety environmental conditions.

Dissertation Organization

This dissertation is organized into six chapters, the first being a general introduction. The second chapter provides an overview of current and past literature important for understanding the basis of the following chapters. Chapter three has been submitted to *Environmental Microbiology* and is a compilation of work focused on exploration of additional exopolysaccharides (EPS) within *P. putida* mt2 biofilm. Here, we described a cellulose-like polymer and the discovery of a novel exopolysaccharide we named putida exopolysaccharide A (Pea) and their contribution to biofilm formation, rhizosphere colonization, and the influence of high osmolarity and water-limitation (or solute and matric potential) on EPS gene expression and production.

In chapter four we focus on the discovery of a novel amyloid-like protein we have named purlin, which contributes to cell-abiotic surface attachment, biofilm stability, and its possible interactions with various EPS components. We also explore how *psgA* and *alg8* gene expression patterns parallel one another and the influence of Bcs and *psgA* mutations lead to increased alginate gene expression based on transcriptional fusion assays.

The fifth chapter provides the first genome-wide transcriptomics study directly comparing *P. putida* gene expression changes to reductions in water availability imposed by sudden decreases in solute or matric potentials. Results of this research provide critical insights into the similarities and differences of matric and solute stress on cell physiology, as well as, identify novel matric stress responses not previously described.

The last chapter summarizes our general conclusions and provides a forum to outline possible future directions on which to expand upon my work. Several appendices follow: Appendix A includes all supplementary information for chapter three such as, a list of PCR primers used in chapter two, results from rhizosphere colonization assay, a figure of complementation assays of biofilm formed on glass coverslips, reaction of *P. putida* and *P. aeruginosa* crude EPS with anti-Psl antisera, PCR verification of EPS mutants. Appendix B contains results of fatty acid analysis of EPS and puril mutants during growth in water stress and replete conditions. Appendix C includes results from preliminary biofilm phenotype experiments prior to construction of the Δ*bcs*Δ*pea*Δ*psgA* mutant. Appendix D alignments of the *algD* promoter region of *P. aeruginosa* and *P. putida,* as well as a more extensiove comparison between selected Pseduomonas strains. Lastly, appendices E and F contain tables of all genes significantly induced or repressed by matric and solute stress, respectively.

Chapter 2. Literature Review – Hydrate or die: Bacterial tolerance and survival mechanisms used during exposure to low-water content habitats

Water availability

Water is necessary for sustaining life and bacteria often live in diverse habitats that can undergo fluctuations in water availability. Water availability is expressed in terms of water potential (Ψ) and is a quantification of the thermodynamic capability of water relative to pure water. Water potential is generally expressed on the MPa (mega pascal) scale with a more negative number meaning lower water availability (Campbell, 1988). There are numerous factors that contribute to negative water potentials but the two most encountered are the solute potential during saturated water conditions, or a combination of solute and matric potential during low-water content conditions (Harris, 1980). During unsaturated conditions, matric potential is the dominant force. The primary distinction between the two is that during a solute stress outer membrane permeable solutes enter the cell and increases osmotic pressure causing the exodus of water from the cytoplasm. Thus, cells are continuously bathed in water, albeit of lowered water potential (Potts, 1994). Conversely, during matric stress, water is physically sorbed onto membrane impermeable surfaces and water exits the entire cell leaving only a very thin layer of surrounding cells and, hence, reduces its availability to hydrate the cell (Harris, 1980, Potts, 1994). As habitats dry, both matric and solute water potentials decrease as water-soluble solutes become more concentrated. Thus, cells experiencing matric stress must respond to both matric and solute stresses even though the may be experiencing the same thermodynamically equivalent water potential as during a solute stress alone. Together, this suggests that at thermodynamically equivalent water potentials, the component associated with matric factors are more stressful to cells than solute factors.

Effects of lowered water potentials on bacterial cells

A reduction in water potential due to solute stress is simple to overcome relative to matric stress. The presence of water is maintained in high solute environments thereby the main type of damage cells incur is due to the transient exodus of cytoplasmic water or high ion concentrations, if the solute stress is due to increasing ionic concentrations. In contrast, two general outcomes occur upon matric stress exposure: the first is mechanical resulting from the removal of the water from macromolecules, and the second is the downstream damage caused from the first, such as perturbation of enzymatic actions and build-up of toxic by-products. Membranes, proteins, and nucleic acids are damaged by both primary and secondary outcomes of matric stress thus making it difficult distinguish between the two. Response and tolerance to water loss are further complicated by other environmental factors, like nutrient limitations, and the rate at which water loss occurs. Less dynamic responses are required during slow reductions in water availability, giving cells time to acclimate compared to a sudden decrease in water availability. The structure, physiological and metabolic state, previous life history, and metabolic diversity of a cell also influences its ability to sense the severity of water stress and the tolerance mechanisms it employs.

Experimental approach to studying lowered water potentials

Many factors present within natural environments make isolating solute or matric effects difficult. Solute stress conditions are easy to manipulate in comparison to matric stress. Lowering the water potential using solutes is achieved by amending growth medium with sucrose or outer membrane permeable salts, such as NaCl or K_2SO_4 which, in gram-negative bacteria, concentrates in the periplasm leading to increased osmotic pressure and outward flow of water from the cytoplasmic membrane to re-establish equilibrium across the cell compartments (Kieft *et al.*, 1987). In contrast, matric conditions are more difficult to study and regulate. Soil systems provide the most natural laboratory condition for studying matric stress since saturated soils can be dried to various water potentials (Wilson and Griffin, 1975, Hartel and Alexander, 1986, Kieft *et al.*, 1987, Rattray *et al.*, 1992, Pesaro *et al.*, 2004). However, soil heterogeneity causing uneven wetting and dying complicates

creating uniform water potential. Furthermore, the charges and multiple crevices associated with soil particles make recovery of cells or their macromolecular constituents difficult. To increase homogeneity and recovery rates, soil samples are often sieved to homogenize soil aggregate size, consequently destroying some of their intrinsic soil properties. Alternatively, vermiculite can be hydrated to various water potentials but its heterogeneity, like soils, also compounds desired results (Sharp *et al.*, 1988).

Monitoring microcolony and biofilm development in soil systems is difficult. To overcome this the Porous Surface Model (PSM) method was developed (Dechesne *et al.*, 2008). In the PSM system bacteria are inoculated onto porous ceramic plates saturated in liquid growth medium and water film thickness is altered at the plate surface by suction in proportion to the amount of matric potential desired. Microscopic observation of cells present on the plate surface allows for dissection of individual or biofilm cell responses. To date, this method has been used to track motility and growth rates of the population (Dechesne et al., 2008). The PSM system offers two advantages that soil systems do not: observation of real-time and non-destructive fluctuations of water availability within the same population. However, the major disadvantage to this method is water reductions are limited to -10 kPa; a narrow range considering the wilting point of plants occurs at -1.5 MPa (Richards and Weaver, 1943). Alternatively, a hybrid system of desiccation jars and alteration of water potential by suction forces has been used but also has the disadvantage of being limited to the -20 kPa range (McGovern *et al.*, 2001).

To achieve more negative matric water potentials in the MPa range three methods are used. The first uses a closed glass chamber (desiccation jars) partially filled with a saturated salt solution to lower the relative humidity of the system and desiccate contents within, including cells (Winston and Bates, 1960, Mary *et al.*, 1986, Chang *et al.*, 2004). This system overcomes the heterogeneity associated with soil systems but precise control and measurement of water content is difficult. Furthermore, once the proper relative humidity is achieved, the system must remain a closed system and incubated at constant temperature since outside conditions can alter relative humidity (Winston and Bates, 1960). Secondly, dehydrating liquids, such as ethanol, can penetrate into the cytoplasm and

displace water. Ethanol-induced dehydration can cause similar effects as other dehydration methods, such as stimulation of alginate production, but have the disadvantage of disrupting membrane structure, rendering the experimental results difficult to interpret (DeVault *et al.*, 1990, Hallsworth *et al.*, 2003, Bagge *et al.*, 2004). Lastly, membrane impermeable desiccation agents can be used such as the non-ionic and inert polymer, Polyethylene glycol M.W. 8000 (PEG). Altering growth medium with various concentrations of PEG lowers the water potential by binding water, reducing its availability to microorganisms without reducing exogenously provided nutrients (Chang *et al.*, 2007, Bester *et al.*, 2010).

Tolerance mechanisms

As water exits out of the cell it shrinks causing loss of turgor pressure and increasing molecular crowding (Poolman and Glaasker, 1998, Minton, 2001). Aggregation of proteins, condensation of nucleic acids, and membrane distortions lead to reduced bacterial fitness (Potts *et al.*, 1987, Potts, 1994, Zonia and Munnik, 2007). To abate these negative consequences, cells must respond quickly, efficiently, and coordinate multiple stress responses. Tolerance relies on multiple protection factors such as osmoprotectants, modification of lipid components, use of heat shock proteins and proteases, antioxidants, and biofilm exopolymeric substances. These factors may be pre-existent or synthesized in response to water stress events. Since intracellular water potential adjustments and the physical effects of osmotic pressure on the cell membrane are similar during solute and matric stress we can extrapolate some of the scientific findings associated with cell responses to solute stress to matric stress responses. However, unique matric stress responses also exist since during water physically lost during matric conditions and cell become physically dehydrated while this does not occur during hyper-solute conditions.

Osmoprotectants and Hydrophilins

Reduction of intracellular water content can lead to loss in turgor pressure and a reduction in cell volume (Whalley *et al.*, 1998, Cayley and Guttman, 2000). Upon exposure

to osmotic upshock bacteria can import potassium ions at the expense of its counterion, glutamate, using various transport systems, such as the Kup, Trk, and Kdp systems (Csonka, 1989, Suh *et al.*, 1999, Dominguez-Ferreras *et al.*, 2009). Desiccation also induces gene expression of a potassium ion uptake transporter in *B. japonicum* (Cytryn *et al.*, 2007). Potassium restores cell turgor pressure but can also be toxic at high concentrations (Epstein, 2003). Since potassium uptake is generally the first response this may explain why potassium concentrations can also serve as a second messenger and can trigger uptake or *de novo* synthesis of osmoprotectants (Booth and Higgins, 1990, Prince and Villarejo, 1990, Epstein, 2003, Balaji *et al.*, 2005). Osmoprotectants are preferable to potassium ions since they are non-toxic and can accumulate to high levels without adversely affecting cellular metabolism or structure (Kurz *et al.*, 2010). Many theories have been proposed how osmoprotecants function but the most well known, and generally accepted, is the water replacement hypothesis, in which hydrophilic osmoprotectants serve in the same capacity as water (Clegg *et al.*, 1982).

Besides osmoprotectants, high-glycine content hydrophilic proteins, known as hydrophilins, also protect and stabilize macromolecules. Furthermore, they have been implicated in desiccation tolerance in both bacterial and eukaryotic cells (Garay-Arroyo *et al.*, 2000, Goldgur *et al.*, 2006, Tolleter *et al.*, 2007). Two groups of hydrophilins exist, those that were, as they were originally attributed to the late embryonic abundant (LEA) proteins accumulated in plant seeds during desiccation, and those generally referred to as hydrophilins with a KGG motif. (Close, 1996, Zhang *et al.*, 2000). Putative hydrophilin genes are expressed in *E. coli* and some are regulated by RpoS (Garay-Arroyo *et al.*, 2000, Robbe-Saule *et al.*, 2007). *P. aeruginosa* expresses three hydrophilin-encoding genes in response to osmotic stress (Aspedon *et al.*, 2006). It is unclear what the exact mechanism of hydrophilin usage is and if they require an association with pre-existing osmoprotectants (Schembri *et al.*, 2004, Tunnacliffe and Wise, 2007). It is also unknown if hydrophilins are present in all compartments of the bacterial cell or exclusive to the cytoplasm. In plants, one LEA-like protein locates to the plasma membrane suggesting hydrophilins can aid membrane cell components susceptible to desiccation (Sales *et al.*, 2000).

Lipid Membranes

Loss of water causes packing of membrane lipids since water occupies space between fatty acid tails and lipid head groups (Gawrisch *et al.*, 1992, Odumeru *et al.*, 1993, Hsieh *et al.*, 1997). Membranes can become viscous due to water loss creating a liquid-to-gel phase transition (Seelig and Seelig, 1980, Hui and Sen, 1989, Crowe *et al.*, 1992). Cells can overcome reduced membrane fluidity by altering their fatty acid structure, phospholipid head groups, or remove lipids completely (McKersie and Stinson, 1980, Potts *et al.*, 1987, Kieft *et al.*, 1994, Boumahdi *et al.*, 2001). In *P. putida* lysophospholipids, phospholipids that have one fatty acid tail, accumulate with decreasing matric water potential and is accompanied by the production of lysophospholipases to remove these potentially toxic lipids (Chang *et al.*, 2004, van de Mortel, 2005). Vitrification of non-reducing sugars (i.e. trehalose and sucrose) associated with phospholipids can help prevent membrane rigidification needed for desiccation tolerance (Crowe *et al.*, 1992, Leslie *et al.*, 1994, Sun *et al.*, 1994, Hoekstra *et al.*, 1997, Wolfe and Bryant, 1999). Lipids are not the sole constituent of membranes and the cell envelope as a whole contains many proteins responsible for sensing environmental signals, eliciting downstream regulatory events, and numerous metabolic functions.

Protein damage

Although water has not been thought of as a co-factor per se, water is needed to hydrate enzyme active sites and retain protein structure flexibility and activity (Clark Douglas *et al.*, 1989, Sage *et al.*, 1996, White and Wimley, 1999). Proteins embedded within or associated with the cell membrane are at elevated risk of denaturation due to their direct contact with the environment. Desiccation causes protein aggregation, exposure of hydrophobic regions and subsequent enzymatic inactivation, and changes in tertiary architecture that prevents their use as structural proteins (Crowe *et al.*, 1987, Allison *et al.*, 1999, Tarek and Tobias, 2002). Increased protease production or activity selectively removes unsalvageable proteins, while chaperons assist protein folding. Clp family

proteases are implicated in the participation of multiple stress responses suggesting they are key to bacterial fitness (Ekaza *et al.*, 2001). Many of these chaperones were identified previously as heat shock proteins (HSP) since protein gels determined their unique synthesis occurred after cells were exposed to sudden elevated temperatures. The σ^H regulon controls production HSP along with many other stress response proteins, 25 % of which are estimated to be located in the cell envelope (Straus *et al.*, 1987, Manzanera *et al.*, 2001, Nonaka *et al.*, 2006). The term 'heat shock proteins' remains but these proteins have broader roles than heat shock alone (Neidhardt *et al.*, 1984, Georgopoulos and Welch, 1993).

Protein damage within the cell envelope can elicit the synthesis or release of several extracytoplasmic function (ECF) sigma factors responsible for global gene expression changes (Missiakas and Raina, 1998). Indirect evidence suggests that the AlgU sigma factor becomes active during matric, but not solute stress, underscoring one of the major differential effects between the two stresses (Chang et al., 2007). AlgU is a homolog σ^E in *E. coli* and activates a well documented 'envelope stress response'. Other ECF factors are also present but little is known about their specific functions since many of the downstream pathways they regulate can overlap with other regulatory systems (Missiakas and Raina, 1998, Heimann, 2002). Two-component regulatory systems also relay information about the status of the cell envelope inward to the cytoplasm where regulatory changes in gene expression can occur. The GacS-GacA is one two-component system that regulates a wide variety of physiological processes and increases accumulation of the σ^S stationary phase sigma factor (Whistler *et al.*, 1998, Workentine *et al.*, 2009). Together, ECF sigma factors and two-component systems are the primary regulators that sense envelope protein changes and respond by stimulating broad, overlapping regulatory changes.

Oxidative stress

Damage to envelope proteins, especially those within the electron transport chain, reduces their efficiency causing sloppy electron transfer and increased free radical

production (Privalle and Fridovich, 1987, Sikora *et al.*, 2009). Reactive oxygen species (ROS) or reactive nitrogen species (RNS) are generated from the reduction of molecular oxygen or nitric oxide, respectively and both can oxidize protein, protein co-factors, lipids, and nucleic acids (Teebor *et al.*, 1988, Halliwell and Gutteridge, 1989, Fang, 2004). ROS and RNS can be extremely detrimental to bacterial growth and eukaryotic hosts often produce these as microbial biocides (Bell *et al.,* Fang, 2004). Proteins and DNA regions that bind iron are especially prone to oxidation damage due to Fenton chemical reactions (Imlay *et al.*, 1988, Lloyd *et al.*, 1997). Antioxidants, slowing metabolic activity, shifting to free radical-tolerant enzymes, and use of ROS scavenging enzymes, such as catalase and perioxidases, are all ways in which cells can defend against the toxic effects of reactive oxygen and nitrogen species (Anderson, 1998, Elkins *et al.*, 1999, Mailloux *et al.*, 2009, Cabiscol *et al.*, 2010). The oxidative stress response is complex but generally involves the SoxRS and OxyR transcriptional factors in *E. coli* (Pomposiello and Demple, 2001). *P. aeruginosa* OxyR mutants are hyper sensitive to hydrogen peroxide and is involved in the induction of the *katB* catalase gene, however, the oxidative stress response is not dependent on the SoxR response regulator since no SoxS sensory protein has been found (Ochsner *et al.*, 2000, Palma *et al.*, 2005). Microarray data suggests the oxidative stress response in *E. coli* overlaps with the heat shock response and the SOS response elicited during DNA damage underscoring the complexity of the oxidative stress response (Zheng *et al.*, 2001). However, the best way to prevent free radical damage is to retard or never be exposed to conditions favorable for radical species formation. Thus, placing a barrier between the cells and the external environment by forming biofilms may be the best first-line defense against exposure to harsh environmental factors (Lewis, 2001, Teitzel and Parsek, 2003).

Biofilms

Biofilms are defined as cells adhered to a surface enmeshed within a matrix of their own making. Biofilm cells are more resistant to environmental stresses and antimicrobial compounds than planktonic cells (Chang et al., 2004, Johnson, 2008). The heterogenous nature of biofilms can also increase population diversity since each individual cell

experiences different environmental conditions than another cell in close proximity (Costerton *et al.*, 1994, Bester *et al.*, 2010, Fey, 2010, Perez-Osorio *et al.*, 2010, Veening and Kuipers, 2010). Protection is afforded to biofilms cells on two non-mutually exclusive levels: at the mechanical and genetic levels. On the mechanical level, cells are surrounded by exopolymeric substances that contain exopolysaccharides (EPS), proteins, lipids, DNA, and other cells that form a physical barrier between an individual cell and the surrounding milieu (Costerton *et al.*, 1995, Zhang *et al.*, 1998, Steinberger and Holden, 2005). On the genetic level, cells that sense environmental stress can trigger multiple signal transduction pathways or guanine cyclases to produce a second messenger molecule, cyclic-di-GMP, leading to production of protein adhesins and exopolysaccharides (EPS) used in biofilm development (Borlee *et al.*, 2010, Guo and Rowe-Magnus, 2010, Krasteva *et al.*, 2010, Lamprokostopoulou *et al.*, 2010). Cyclic-di-GMP also regulates a multitude of other metabolic and physiological functions, suggesting adhesin and EPS production are co-regulated by overlapping, highly integrated, post-secondary regulatory networks (D'Argenio and Miller, 2004, Jenal and Malone, 2006, Weber *et al.*, 2006). Some *Pseudomonads* contain the large adhesin protein, LapA, which is similar in function to the *Salmonella* Bap and *E. coli* YeeJ adhesins (Latasa *et al.*, 2005, Monds *et al.*, 2007). Self-aggregating proteins like curli, tafi, fimbriae, and others can serve as adhesin connecting cells to the surface or one another (Prigent-Combaret *et al.*, 2000, Wang *et al.*, 2004, Ledeboer *et al.*, 2006).

Most biofilm studies occur in water-saturated conditions, however, this is not the norm for cells in terrestrial habitats. In water-limited environments, such as in the phyllosphere, where water fluctuations are predominate, cells are typically found in small aggregates, referred to as microcolonies, which then form into a more populated, mature bioiflm (Lindow and Brandl, 2003). Under water-limiting conditions hygrospoic EPS, such as the alginate regulated by AlgU, can aid in hydration of cells and prevent further water loss (Bagge et al., 2004). In fact, alginate retains enough water that up to an estimated one-third of water-soaked leaf weight was attributed to the presence of microbial alginates (Morse, 1990, Denny, 1995, Sutherland, 2001, Chapotin *et al.*, 2003). Multiple EPS polymers can usually be produced by a single organism. *P. aeruginosa* contains Psl, a mannose-

galactose rich EPS polymer, and Pel, a glucose-rich EPS in addition to alginate which highlights the mechanical and versatile roles EPS plays in cell physiology, lifestyle, and mechanical abilities (Jackson *et al.*, 2004, Vasseur *et al.*, 2005, Aspedon *et al.*, 2006, Chang *et al.*, 2007). Psl and Pel exopolysaccharide synthesis is increased with elevated cellular concentration of cyclic-di-GMP generated by guanine cyclases containing GGDEF-domains (Hickman *et al.*, 2005, Cotter and Stibitz, 2007, Lee *et al.*, 2007, Hickman and Harwood, 2008, Nakhamchik *et al.*, 2008). Some GGDEF-domain proteins are induced by water stress and entry into stationary phase (Aspedon et al., 2006, Weber et al., 2006).

Loss of protein adhesins or EPS components can decrease biofilm formation and alter the natural architecture that is associated necessary for optimal bacterial fitness (Danese *et al.*, 2000, Chang *et al.*, 2007, Li *et al.*, 2010). Initially, many adhesins were thought to have a role only during cell attachment to surfaces or each other, but complex models are now emerging in which extracellular proteins and EPS associate together to promote specific cell-cell interactions to stabilize the biofilm. For example, loss of either cellulose or tafi fibers in Salmonella lead to decreased biofilm formation, long-term desiccation tolerance, and resistance to hypochlorite (Aspedon et al., 2006). Loss of both cellulose and tafi fibers reduced biofilm formation and desiccation tolerance more than the loss of each one independently. In *P. fluorescens* cellulose, LPS, and an unknown adhesin protein were all necessary for biofilm formation and in *P. aeruginosa* the haemagglutin protein, CdrA, directly interacts with Psl EPS and is necessary for biofilm stabilization (Spiers and Rainey, 2005, Borlee et al., 2010). Biofilm stabilization in *P. putida* requires the interaction between a cellulase sensitive EPS and the cyclic-di-GMP regulated adhesin protein, LapA (Gjermansen *et al.*, 2010).

Clearly, the biofilm matrix is more structured than previously thought. Key associations between protein and EPS are likely important for optimizing survival during a plethora of stresses, including water stress. Responses to water stress, like many other stresses, require the vast coordination of regulatory, metabolic, and physiological events. Many stress response pathways overlap adding to the difficulty of parsing out which responses are unique to matric stress conditions. However, it is these overlaps that may

make bacteria so adaptable to harsh environmental conditions since induction of one stress response pathway can cross-protect against another stress (Flahaut *et al.*, 1997, Mongkolsuk *et al.*, 1997, Begley *et al.*, 2002). Further complications arise when the previous life history of the cell, biofilm cell heterogeneity, and the additional cellular damage occurs with water loss. Recent studies have added to our knowledge of detailed aspects of biofilm formation, stress responses, and cell-cell interactions that are vital to environmental stress tolerance. However, we are far from understanding what occurs during water stress on the population and community levels in terrestrial habitats. To do so, we must continue to dissect the distinctive properties associated with water stress so we can capitalize on the spectrum of strategies employed during matric stress conditions. The objective this dissertation is to present and summarize studies used to identify additional components of the *P. putida* biofilm and determine what, if any, role they have in water stress tolerance. Also, we seek to define transcriptional differences between osmotic and matric shocked cell since little is known about the initial responses of cells to a sudden reduction in water potential and whether responses are similar for the solute and matric components.

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Chapter 3. Cell-cell and cell-surface interactions mediated by cellulose and a novel exopolysaccharide contribute to *Pseudomonas putida* biofilm formation and fitness under water-limiting conditions

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Summary

The composition of the exopolysaccharide matrix of *Pseudomonas putida* mt2 biofilms is relatively undefined as well as the contributions of each polymer to ecological fitness. Here, we describe the role of two putative exopolysaccharide gene clusters, putida exopolysaccharide A (*pea*) and bacterial cellulose (*bcs*) in biofilm formation and stability, rhizosphere colonization, and matrix hydration under water-limiting conditions. Our findings suggest that *pea* is involved in the production of a novel glucose, galactose, and mannose-rich polymer that contributes to cell-cell interactions necessary for pellicle and biofilm formation and stability. In contrast, Bcs plays a minor role in biofilm formation and stability; although it does contribute to rhizosphere colonization based on a competition assay. We show that *pea* expression is highly induced transiently under water-limiting conditions but only slightly by high osmolarity, as determined by qRT-PCR. In contrast, both forms of water stress highly induced *bcs* expression. Cells deficient in making one or more exopolysaccharide experienced greater dehydration-mediated cell-envelope stress, leading to increased alginate promoter activity. However, this did not lead to increased exopolysaccharide production, except in alginate-deficient mutants, indicating that when unable to synthesize alginate, *P. putida* compensates by producing more exopolysaccharides, presumably to facilitate biofilm hydration. Collectively, the data

suggests that Pea and Bcs contribute to biofilm formation and in turn their presence contributes to fitness under water-limiting conditions, but not to the extent of alginate.

Introduction

Bacterial biofilms are highly structured assemblages of cells that share many secreted molecules, including exopolysaccharides (EPS), proteins, and DNA that collectively comprise the extracellular matrix (Borlee et al., 2010). This matrix is thought to provide multiple functions for the community, serving as a scaffold holding biofilm cells together and providing protection from some antimicrobials, phagocytosis, and environmental stresses. Exopolymer production is highly regulated and is likely influenced by environmental conditions, particularly if the product is a fitness determinant. For example, under water-limiting conditions *Pseudomonas putida*, and other fluorescent Pseudomonads, produce the EPS alginate to facilitate maintaining biofilm hydration and proper biofilm architecture, protecting residents from desiccation stress and increasing survival (Chang et al., 2007). In contrast, alginate is not required for wild type *Pseudomonas aeruginosa* biofilm formation in flow-through systems (Wozniak *et al.*, 2003), were water abundance is not limiting.

Understanding the regulation of matrix component expression is an area of intense interest. Recent reports suggest that cyclic diguanylate (c-di-GMP) positively modulates production of matrix components at the transcriptional and allosteric level for Pseudomonads and other Gram-negative species (Tischler and Camilli, 2004, Hickman *et al.*, 2005, Romling *et al.*, 2005). The developing model is that high intracellular levels of c-di-GMP promote the biofilm lifestyle through matrix production, while lower levels promote motility and the planktonic lifestyle. In *P. aeruginosa*, studies of the Wsp signal transduction system suggest that the production of c-di-GMP is stimulated in response to growth on a surface (Guvener and Harwood, 2007)and mutations in this sensory system can lead to variants with altered EPS production and biofilm properties including small colony variants (Spiers *et al.*, 2003, Starkey *et al.*, 2009). Activation of WspR results in the synthesis of c-di-GMP, increased cellular levels of c-di-GMP, and increased biofilm formation because of

increased expression of Pel and Psl polysaccharides (Hickman et al., 2005) and a secreted adhesion (CdrA) that binds to the Psl polysaccharide to promote auto-aggregation (Borlee et al., 2010). Additionally, in *P. fluorescens* and *P. putida,* c-di-GMP levels influence expression of the outer membrane associated adhesin LapA (Monds et al., 2007), which in *P. putida* appears to be associated with a cellulase-sensitive polylsaccharide (Gjermansen *et al.*, 2010). Similarly, in enteric bacteria increased cellular levels of c-di-GMP can lead to allosteric activation of cellulose synthase and increased cellulose production as well as increased production of proteinaceous appendages called curli fibrils (Kader *et al.*, 2006). Some evidence suggests that interactions between curli and cellulose fibrils facilitate matrix stability and cell-cell interactions (De Jong *et al.*, 2009). Thus, emerging evidence indicates the biofilm matrix is much more complex and ordered than previously suspected and that individual components can be dispensable under certain environmental conditions.

We and others have hypothesized that the composition of the EPS component of the biofilm matrix influences environmental stress tolerance, including desiccation tolerance (Aspedon *et al.*, 2006, Chang *et al.*, 2007). Previously, we showed that alginate production by *P. putida,* and other fluorescent pseudomonads, influences matrix physicochemical properties and biofilm development (Chang et al., 2007). By maintaining biofilm hydration, alginate production may attenuate dehydration-mediated oxidative stress which, if extensive, can lead to cell death (Chang *et al.*, 2009). The fitness advantages associated with alginate production under water-limiting conditions likely explains why most biofilm residents transiently express alginate biosynthesis genes immediately following exposure to a desiccation event (Gjermansen *et al.*, 2010). The importance of alginate in the ecological fitness of *P. putida* needs to be viewed with the understanding that it is not the primary EPS component under water-limiting conditions (Chang et al., 2007).

At present it is unclear what other EPS polysaccharides are synthesized by *P. putida* and their role in unsaturated biofilm development and environmental stress tolerance. In this report, we explore the role of two putative EPS identified in the genome sequence of *P. putida* KT2440 (Nelson *et al.*, 2002) on biofilm development and begin to explore their role in *P. putida's* ecological success in various habitats and under various environmental

conditions. We show that a novel EPS locus that we refer to as putida exopolysaccharide A (*pea*) is important for cell-surface interactions and for maintaining cell-cell interactions post-attachment on abiotic surfaces. Also, we show that the operon for the biosynthesis of a cellulose-like polymer is important for biofilm maintenance post-attachment, rhizosphere fitness, and that its presence may interfere with interactions between the surface and an unidentified surface adhesin. Lastly, we provide data suggesting that both Pea and the cellulose-like polymer may contribute to facilitating biofilm matrix hydration under water-limiting conditions, although not to the extent that alginate does.

Results

Loci potentially involved in EPS production

The cellulose operon (Table 1) is predicted to be comprised of ten genes and includes homologs of components known to form cellulose synthase complexes, a PilZ c-di-GMP binding domain, and transport, based on BLAST analysis and genome annotation (Nelson et al., 2002). PFAM predictions suggest that the BcsQ protein contains a MinD/ParA binding domain implying that cellulose production machinery may be located at the cell poles, as in *E. coli* (Quere and Ghigo, 2009). A cellulase (*bcsZ)* is likely encoded within the operon and is the only predicted cellulase in the *P. putida* KT2440 genome.

Analysis of the *putida exopolysaccharide A (pea)* locus revealed at least six transcriptional units comprised of ORFs predicted to encode proteins involved in the synthesis and transport of polysaccharides (Table 1). We focused on the fifth transcriptional unit (PP3133-3141) containing five putative glycosyltransferases and other genes for polysaccharide modification for generating mutants (Table 1). We used the Carbohydrate-Active enZYmes database to predict potential substrates of these glycosyltransferases and found that *peaI* encodes a putative mannosyl transferase, while PeaH and PeaC are members of group 2, family 2 glycosyltransferases that can donate mannose, rhamnose, N-acetylglucosamine, N-acetylgalactosamine, or glucose to the developing polysaccharide polymer. PeaG is a group 2, family 4 glycosyltransferase that can donate GDP-Man-α-mannose, α-glucose, UDP-Gal-α-galactose, or xylose. We did not

identify any PilZ, GGDEF, EAL, or HD-GYP domains in the *pea* loci, based on PFAM/COG annotation and manual BLAST comparisons with protein sequences demonstrated to be involved in c-di-GMP synthesis, degradation, or binding.

Table 1. *P. putida pea* and *bcs* ORFs with proposed roles in polysaccharide synthesis or export

Gene	ORF	Oper-on[a]	PFAM/TIGR/COG prediction, predicted function, protein similarity (% positive)
			Cellulose (bcs) Operon
bcsD	PP2629	1	Putative protease, *Pectobacterium* sp. BcsE (45-48%)
bcsE	PP2630	1	TIGR03369 bacterial cellulose synthase E, putative protease, *E. coli* 61%
bcsF	PP2631	1	TIGR03493, *Pectobacterium wasabiae* BcsF (52%)
bcsG	PP2632	1	TIGR03368, cellulose synthase operon protein *P. wasabiae* YhjU (63%)
bcsH	PP2633	1	Unknown (Hypothetical)
bcsQ	PP2634	1	PF01656, cellulose synthase containing putative MinD/ParA domain
bcsA	PP2635	1	PF00535, cellulose synthase catalytic subunit, contains PilZ domain, *E. coli* (72%)
bcsB	PP2636	1	PF03170, cellulose synthase regulator protein BcsB, *Pectobacterium carotovorum* (67%)
bcsZ	PP2637	1	PF01270, endo-1,4-D-glucanase/ cellulase, *P. carotovorum* (66%)
bcsC	PP2638	1	PF05420, cellulose synthase subunit C, *E. coli* (56%)
			Putida exopolysaccharide A (pea) locus
	PP3126	1	PF02563, polysaccharide export, pslD PA2234 (47%)
	PP3127	2	PF02706, exopolysaccharide transport/ Wzz chain length determination
	PP3128	2	TIG01007, EPS/capsule, ATPase for chromosome partitioning
galE	PP3129	3	UDP-glucose-4-epimerase
	PP3130	3	β-glycanase based on protein structural homology[b]
	PP3131	3	Hypothetical
	PP3132	4	PF01943, polysaccharide biosynthesis, involved in export of LPS O-antigen
peaA	PP3133	5	PF05199, GMC oxidoreductase, putative choline dehydratase
peaB	PP3134	5	COG0110, acteyltransferase; structural homology to galactoside-acetyltransferase protein
peaC	PP3135	5	PF00532, periplasmic binding protein/Glycosyltransferase group 2, family2[c]
peaD	PP3136	5	TIGR01172, serine O-acetyltransferase, cysE family

[a] Predicted operons according to www.pseduomonas.com

[b] Protein structure homology predicted by Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/)

[c] Glycosyltransferase families according to www.CaZY.org

Table 1. (continued)

Gene	ORF	Oper-on[a]	PFAM/TIGR/COG prediction, predicted function, protein similarity (% positive)
peaE	PP3137	5	PF00535, glycosyltransferase, group 2, rhamnose glycosyl transferase, pslC PA2233 (39%)
peaF	PP3138	5	PF04393, VirK domain, PSEEN2448 *P. entomophila* glycosyltranferase (77%)
peaG	PP3139	5	PF00534, Glycosyltransferase group 1, family 4, PA1391 (48%)
peaH	PP3140	5	PF00535, Glycosyltransferase group 2, family 2
peaI	PP3141	5	PF03808, Glycosyltransferase group 2, family 26, likely involved in mannose transfer
	PP3142	6	PF0239, Sugar Transferase, possible glucose glycosyl transferase, PA2231 PslA (55%)

peaGHI and bcsQAB mutants are deficient in congo red binding and pellicle formation

To assess whether the *pea* or *bcs* loci play a role in biofilm formation we utilized standard reverse genetic strategies to create non-polar deletions in *bcsQAB*, *peaGHI*, and alginate (*algD*) genes in wild type *P. putida* mt2 (see Experimental Procedures and Table 2). The Km-resistance marker was removed for constructing pyramided deletions as described in the Experimental Procedures.

Table 2. Strains and plasmids used in this study

	Relevant characteristic	Source or reference
Strains		
E. coli TOP10	Host for cloning	Invitrogen
P. putida mt2	Wild-type, spontaneous Rf^R	(Chang et al., 2004)
Δ*algD*	*algD* in frame deletion, Rf^R	(Chang et al., 2009)
Δ*peaGHI*	*peaGHI* in frame deletion, Rf^R	This Study
Δ*peaGHI*-Km	*peaGHI* in frame deletion with FRTKm-cassette, Km^R Rf^R	This Study
Δ*bcsQAB*	*bcsQAB* in frame deletion, Rf^R	This Study
Δ*bcsQAB*-Km	*bcsQAB* in frame deletion with FRTKm-cassette, Km^R Rf^R	This Study
Δ*pea*Δ*alg*	Δ*peaGHI* with *algD* in frame deletion, Rf^R	This Study
Δ*bcs*Δ*alg*	Δ*bcsQAB* with *algD* in frame deletion, Rf^R	This Study
Δ*pea*Δ*bcs*	Δ*bcsQAB* with *peaGHI* in frame deletion, Rf^R	This Study
Δ*pea*Δ*bcs*-Km	Δ*bcsQAB* with *peaGHI* in frame deletion with FRTKm-cassette, Km^R Rf^R	This Study
Δ*pea*Δ*bcs*Δ*alg*	*ΔbcsQABΔpeaGHI* with algD in frame deletion, Rf^R	This Study
P. aeruginosa PAO1	Wild-type	M. Parsek

Table 2. (continued)

	Relevant characteristic	Source or reference
PAO1 Δ*pslBCD*	PAO1 with *pslBCD* deletion	M. Parsek
Plasmids		
pGemT-Easy	Cloning vector, Ap^R	Promega
pGbcs-FRTKm	pGemT-Easy with Δ*bcsQAB*-FRTKm cassette, Ap^R Km^R	This Study
pGpea-FRTKm	pGemT-Easy with Δ*peaGHI* -FRTKm cassette, Ap^R Km^R	This Study
pEX18Tc	allelic exchange vector, Tc^R, SacB	(Hoang *et al.*, 1998)
pEXI18-*algD*	algD allelic exchange vector , Tc^R Km^R	(Chang et al., 2009)
pEX*pea*	peaGHI alleclic exchange vector, Tc^R Km^R	This Study
pEX*bcs*	bcsQAB allelic exchange vector, Tc^R Km^R	This Study
pKD4	vector carrying Km-FRT cassette	(Buck *et al.*, 2000)
pFlp2	encodes Flp recombinase, Ap^R	(Hoang et al., 1998)
pME6041	shuttle vector, Km^R	(Heeb *et al.*, 2000)
pMe-Pea	pME6041 containing Δ*peaGHI* genomic fragment, Km^R	This Study
pMe-Bcs	pME6041 containing Δ*bcsQAB* genomic fragment, Km^R	This Study
pVSP61	expression vector, Km^R	(Spiers et al., 2003)
pWspR19	constitutively active WspR19 allele, Km^R	(Spiers et al., 2003)
miniTn7gfp_{AAV}	chromosomal gfp marker, Gm^R	(Lambertsen *et al.*, 2004)

Studies on the genetic basis of biofilm formation have been greatly facilitated by the development of simple phenotypic assays, including colony morphology (Bokranz *et al.*, 2005), pellicle formation (Friedman and Kolter, 2004), congo red and calcofluor binding (Spiers et al., 2003), and quantification of biofilm formation on solid surfaces by crystal violet staining (O'Toole and Kolter, 1998) or microscopy (Vasseur et al., 2005). Wild type mt2 did not form robust pellicles or exhibit strong congo red binding properties. Previous work (Spiers *et al.*, 2003, Hickman *et al.*, 2005) has shown that c-di-GMP can stimulate exopolysaccharide production in a number of bacteria and we wanted to determine whether *pea* and *bcs* expression are stimulated by increased c-di-GMP levels. We reasoned that the constitutively active WspR19 mutant protein was an ideal candidate for increasing

intracellular c-di-GMP pools since WspR increases exopolysaccharide production in other closely related Pseudomonas species (Spiers *et al.*, 2003, Hickman *et al.*, 2005). WspR19 expression by mt2, Δ*algD*, and Δ*bcsQAB* under hyper, but not hypo-osmotic conditions (Fig. 1A) resulted in robust, stable pellicles within 48 h in static cultures. In contrast, the thin pellicles formed by *ΔpeaGHI* collapsed from the center outwards by 24 to 36 h, suggesting Pea mutants are unable to maintain cell-cell interactions necessary for maintaining robust pellicles.

The effect of WspR19 expression by mt2, Δ*algD*, and Δ*bcsQAB* was also apparent in the congo-red binding properties of colonies, which were dark red within 48 h post-inoculation whereas strains containing the Δ*peaGHI* deletion were faint pink (Fig. 1B). None of the strains developed wrinkled colonies as reported for *Salmonella, P. aeruginosa,* and *P. fluorescens* (Zogaj *et al.*, 2001, Friedman and Kolter, 2004, Spiers and Rainey, 2005). Notably, a second congo red binding matrix component was visible in strains with the Δ*peaGHI* deletion after 4 to 7 days of growth (Fig. 1B).

peaGHI and bcsQAB mutants are defective in biofilm formation

To determine if Pea or Bcs genes have a role in biofilm formation we employed biofilm microtiter dish and glass coverslip assays. Adherence properties of mt2 and EPS-deficient mutants to microtiter wells were similar to each other during early stages of biofilm formation (6 h). However, over time Δ*peaGHI* and Δ*bcs*Δ*pea* biomass substantially decreased relative to mt2 and Δ*bcsQAB* (Fig. 2A). Additionally, unlike mt2, the Δ*bcsQAB* mutant was unable to increase surface-associated biomass at 48 h. These defects were not a consequence of differences in growth or motility since mt2 and the EPS-deficient strains had similar growth rates, and swimming and twitching motility behaviors (data not shown). Furthermore, LPS banding patterns of mt2 and the EPS-deficient mutants were similar (data not shown).

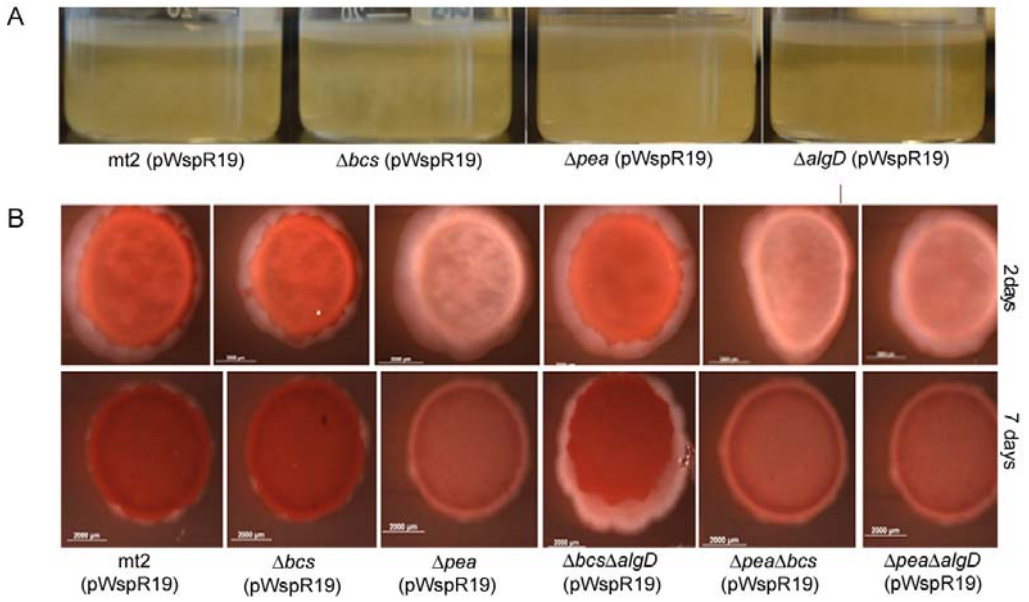

Figure 1. Pellicle and congo red binding phenotypes of *P. putida* WspR19 expressing strains. (A) Pellicle formation in static cultures 48 h after inoculation in TYE broth amended with 0.3M NaCl. (B) Congo red binding phenotypes of colonies on LB agar containing coomassie blue and congo red.

We also assessed biofilm formation on glass coverslips at the air-liquid interface by mini*Tn7gfp*$_{AAV}$-tagged derivatives in the presence or absence of minor fluid shear forces imposed by gentle shaking. Surface area covered by biofilms at the air-liquid interface was measured using the image analysis tools available with the microscope imaging software as described in the Experimental Procedures. Consistent with our microtiter plate assay, in the absence of shaking, biofilms formed on glass coverslips 6 h post-inoculation by Δ*peaGHI* and Δ*bcsQAB* were similar to mt2 but by 24 h mt2 and *ΔbcsQAB* biofilm surface area were still similar to each other yet significantly greater than Δ*peaGHI* (Table 3 and Fig. 2B). Defects in biofilm formation was clearly mediated by the *peaGHI* deletion since introduction of the pMe-Pea complementation plasmid harboring a 3.2 kb insert containing the *peaGHI* locus into *ΔpeaGHI* restored surface area coverage to wild type levels (Fig. 3).

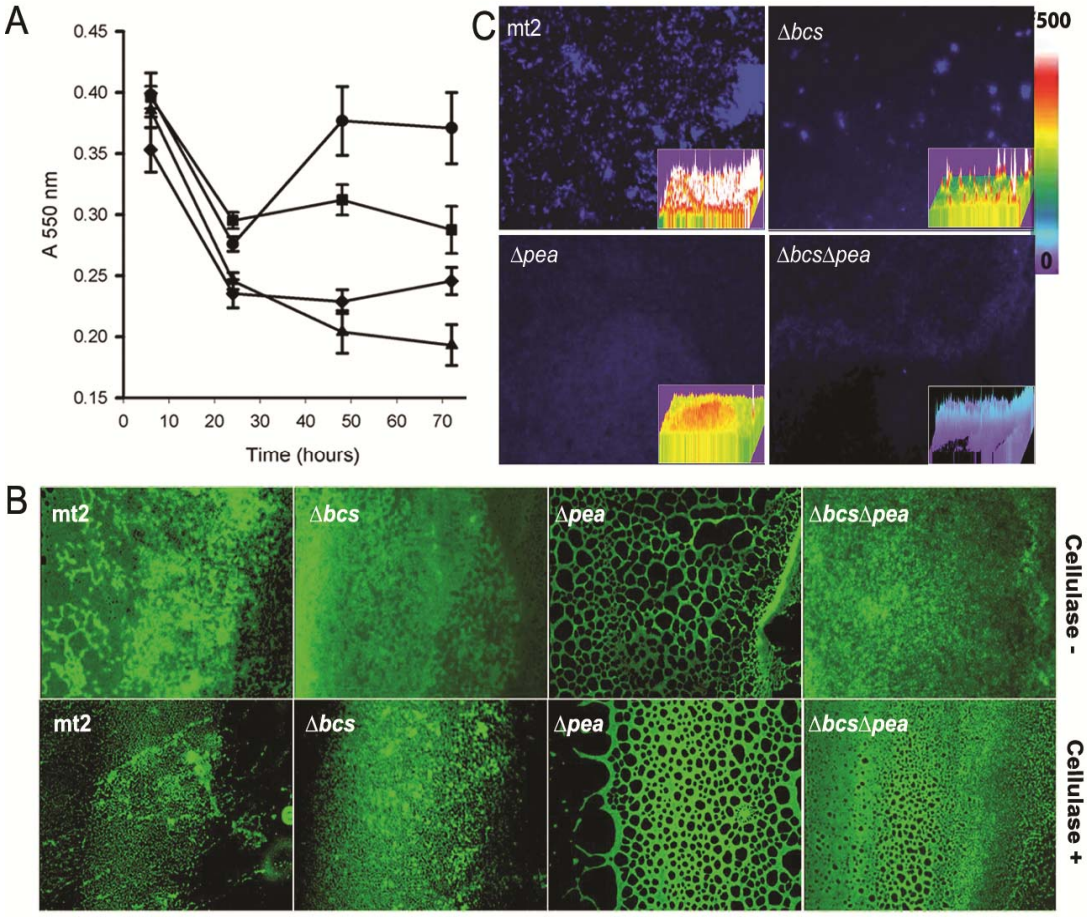

Figure 2. Biofilm formation properties of *P. putida* wild type and EPS-deficient strains. (A) Microtiter plate biofilm assays of mt2 (●), Δ*bcsQAB* (■), Δ*peaGHI* (◆), and Δ*bcs*Δ*pea* (▲). Values are the mean and error bars (SEM) of three or more experiments. (B) Representative images of 24 h old biofilms formed by miniTn7gfp_{AAV} tagged cells at the coverslip air-liquid interface in JM mannose medium without (-) or with (+) 200 U cellulase treatment. A 10x objective lens was used to take images of horizontally-orientated coverslips. (C) Calcofluor staining of 24 h old biofilms at the coverslip air-liquid interface. One day old biofilms were stained in a 100 µM calcofluor solution and rinsed prior to obtaining epifluorescence microscopy images with a 20x objective lens. Inserts are histograms of fluorescence intensity profiles of each pixel after background correction. Sidebar provides the color coded fluorescence intensity values used to derive the fluorescence intensity histogram insets.

However, unlike the microtiter plate biofilm assay, we did not observe a similar decrease in biofilm formation by Δ*bcsQAB* in our glass coverslip assay (compare Fig. 2A and Table 3). Interestingly, biofilms by *ΔbcsΔpea* covered more surface area in the coverslip assay than *ΔpeaGHI* (Fig. 2B). This was due to a defect in Bcs production since introduction of pMe-Bcs, which harbors an insert containing the *bcsQAB* locus, into *ΔbcsΔpea* resulted in

a statistically significant decrease in biofilm formation, although not quite to the levels of *ΔpeaGHI* (Fig. 3). Collectively, the data suggests that the surface properties of *ΔbcsΔpea* exhibits better cell-surface adhering properties than Δ*peaGHI* (Fig. 2B, Fig. 3) but is unable to maintain the cell-cell interactions necessary for increasing biofilm biomass post-attachment (Fig. 2A).

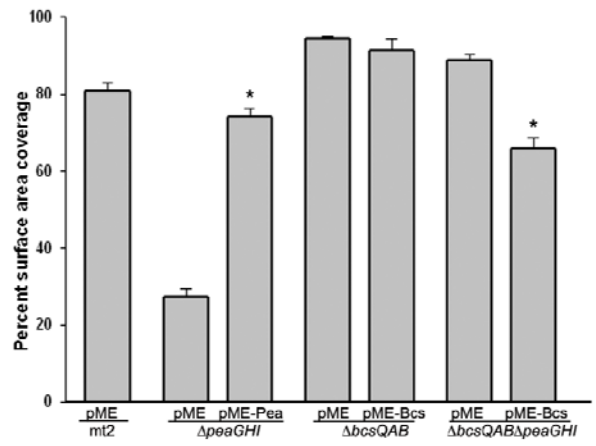

Figure 3. Complementation assays of *P. putida* EPS-deficient mutants. Surface area covered (%) on glass coverslips at the air-liquid interface 24 h post-inoculation by strains containing an empty pME6041 (pME) vector, or complementation vectors pME-Pea or pME-Bcs. Bars represent the mean ± SEM of two experiments, each comprised of 35 replicates. Asterisks indicate there was a statistically significant increase (Δ*peaGHI* (pME-Pea)) or decrease (Δ*bcs*Δ*pea* (pME-Bcs)) in biofilm surface area coverage between the complement and empty vector control (pME), based on a pair-wise comparison ($P<0.05$).

Table 3. Biofilm formation by *P. putida* mt2 and EPS-deficient mutants on glass coverslips at the air-liquid interface under static conditions [a]

	Mean ± STD at the following times (hours)		
Strain	6	24	48
mt2	89.9 ± 5.6	87.6 ± 4.8	53.5 ± 13
Δ*bcsQAB*	91.0 ± 5.2	92.5 ± 3.1	56.4 ± 8.4
Δ*peaGHI*	86.4 ± 8.6	57.7 ± 7.2	48.1 ± 12.7

[a] Values are % surface area covered by biofilm

Pea is a cellulase-sensitive polysaccharide

Cellulose and other polysaccharides can be degraded by cellulases targeting β-1,3 and β-1,4-linked glucans (Emert *et al.*, 1974). To determine if the Pea or Bcs polysaccharides are cellulase-sensitive we employed the coverslip biofilm assay and allowed biofilms to form in the presence or absence of cellulase. Biofilm surface area coverage by mt2 in the presence of cellulase was greatly reduced compared to in the absence of cellulase (Fig. 2B and Table 4), indicating that mt2 biofilms contain one or more cellulase-sensitive components. In contrast, Δ*peaGHI* biofilm surface area coverage was similar in the presence and absence of cellulase (Table 3). Furthermore, biofilm surface area coverage by Δ*bcsQAB* was marginally reduced in the presence of cellulase compared to its absence (Fig. 2B) providing strong evidence that Pea, and not Bcs, is the primary cellulase-sensitive component. The apparent lack of Bcs sensitivity to cellulase may be a consequence of the low amounts of this polymer in the matrix, acetylation, or masking by other matrix components. Furthermore, Pea is apparently not the only cellulase-sensitive component since biofilm formation by Δ*bcs*Δ*pea* was also cellulase-sensitive, albeit only slightly.

Table 4. Effect of cellulase on biofilm formation by *P. putida* strains on glass coverslips at the air-liquid interface.

Strain	Cellulase treatment[a]	Mean % surface area[b]	
mt2	-	77.0	A[c]
mt2	+	31.7	C
Δ*bcsQAB*	-	72.4	A
Δ*bcsQAB*	+	56.3	B
Δ*peaGHI*	-	34.4	C
Δ*peaGHI*	+	37.0	C
Δ*bcs*Δ*pea*	-	73.8	A
Δ*bcs*Δ*pea*	+	65.8	B

[a] Biofilms formed in the presence (+) and absence (-) of 200U cellulase.
[b] Biofilm surface area coverage on glass coverslips at the air-liquid interface after 24 h.
[c] Values followed by the same letter are not significantly different ($P \geq 0.05$, LSD).

Pea and Bcs polysaccharides bind calcofluor

Wild type mt2 biofilms formed on glass coverslips strongly binds calcofluor 24 h post-inoculation (Fig. 2C). However, biofilms by Δ*bcsQAB* exhibited reduced calcofluor binding and had fewer calcofluor bright foci relative to mt2 (Fig. 2C) while biofilms formed by Δ*peaGHI* bound less calcofluor and had even fewer calcofluor bright foci (Fig. 2C). Calcofluor binding by Δ*bcs*Δ*pea* biofilms (Fig. 2C) was extremely poor, suggesting that calcofluor primarily binds the Bcs and Pea polymers, although it is conceivable the other Congo red binding polymer produced as colonies age could bind calcofluor as well.

Bcs but not Pea contributes to maize root colonization

A previous study indicated that alginate expression was a fitness determinate for maize rhizosphere colonization by *P. putida* (Ramos-Gonzalez *et al.*, 2005). Thus, we wanted to assess the potential roles of cellulose and Pea in rhizosphere fitness. We found that Δ*bcsQAB*, but not Δ*peaGHI*, have reduced rhizosphere colonization ability during competition with mt2. The proportion of each strain in the rhizosphere population was determined after 7 days (Appendix A) and compared to the proportion of each strain in the inoculum. In the absence of a wild type competitor, Δ*bcsQAB* and Δ*peaGHI* , but not Δ*algD*, colonized corn roots as well as mt2 (data not shown). Furthermore, Δ*bcsQAB* and Δ*peaGHI* were equally capable as the wild type to attach to corn seed in a short term (2 h) assay. Collectively our data suggests that both Bcs and alginate are important for rhizosphere fitness.

Exo- and capsular- polysaccharide composition analysis

We wanted to determine the chemical composition of the EPS and CPS of biofilms to gain insight into the monomer composition of Pea and Bcs. Both EPS and CPS was isolated from surface grown colony biofilms since it is unclear if Bcs or Pea is loosely or tightly associated with cells. We obtained material from strains with the WspR19 allele since our congo red binding data suggested more EPS was produced by c-di-GMP over expression.

Chemical analyses revealed that monomer composition of the EPS and CPS are similar, except for the absence of xylose in the CPS (Table 5). The primary difference in the carbohydrate content of *ΔpeaGHI* from wild type biofilms was the reduction in mannose and rhamnose and increase in glucose content (Table 5). It's possible that lack of Pea results in increased production of Bcs when c-di-CMP is over expressed by the WspR19 allele. In contrast, the EPS and CPS from Δ*bcsQAB* and mt2 biofilms were fairly similar, except for the increase in the glucose content of Δ*bcsQAB* matrix material, possibly due to increased Pea production. Although the presence of rhamnose could be due to LPS contamination, we did not detect 3-keto-deoxy-d-manno-octulosonic acid in our samples. Given that the *P. aeruginosa* polysaccharide Psl is also comprised of glucose, mannose, and rhamnose (Byrd *et al.*, 2009) and that the *pea* loci has three glycosyltransferases with homology to *psl* glycosyltransferases it's conceivable that the Pea and Psl polysaccharides are structurally similar. To test this we performed Western immunoblotting on crude EPS extracts (see Experimental Procedures) using polyclonal Psl-antisera (Byrd et al., 2009). Psl-antisera detected Psl production by *P. aeruginosa* PAO1 but not a PAO1Δ*pslBCD* mutant and did not cross react with *P. putida* EPS suggesting that Psl and Pea are structurally distinct (Appendix A).

Table 5. Glycosyl composition of EPS and CPS isolated from biofilms formed by *P. putida* WspR19 expressing cells[a].

	Mole % of total carbohydrate					
	EPS			CPS		
Glycosyl residue	mt2	Δ*peaGHI*	Δ*bcsQAB*	mt2	Δ*peaGHI*	Δ*bcsQAB*
Mannose	17.1	10.9	15.3	14.9	8.7	17.8
Galactose	7.9	11.6	10.8	1.9	3.0	nd[b]
Glucose	15.5	38.1	32.8	31.8	56.7	71.7
Xylose	5.3	10.6	7.4	nd	nd	nd
Rhamnose	54.2	31.0	40.1	51.4	31.6	53.9

[a] Biofilms were cultivated for 48h before EPS and CPS isolation. Values represent two independent EPS isolations each containing 3 biological replications that were pooled for analysis.
[b] Not detected.

qRT-PCR of EPS gene expression

Previous work had indicated that alginate gene expression is highly induced under water-limiting but not high osmolarity conditions (Chang *et al.*, 2007, Gjermansen *et al.*, 2010). Thus we were interested in determining whether *bcs* or *pea* expression is also differentially regulated by these two forms of water deprivation. As can be seen in Figure 4A, comparative qRT-PCR analyses of RNA isolated from wild type mt2 biofilms cultivated under high osmolarity or water-limiting conditions showed that relative to water-replete conditions *pea* expression was differentially regulated by these forms of water deprivation in a manner that parallels alginate expression. Sequence analysis indicates the presence of a putative AlgT (σ^{22}) and one AlgR binding site upstream of the *peaI* transcriptional start site with modest sequence similarity to mt2 *algD* promoter region (data not shown). Interestingly, *bcsA* expression was equally induced by high osmolarity and water-limiting conditions (Fig. 4A) in a temporal-independent manner.

EPS-deficient biofilm cells sense more dehydration stress

Given that *peaI* and *bcsA* expression increase under water-limiting conditions (Fig. 4A) we explored whether EPS deficiency leads to increased dehydration stress using our previously described *algD* operon promoter-*gfp* transcriptional fusion (Chang *et al.*, 2007, Gjermansen *et al.*, 2010). Our findings show that a greater proportion of EPS-deficient mutant cells expressed the alginate reporter compared to the wild type under water-limiting (Table 6) but not water-replete conditions (data not shown). The inability to produce two or more EPS did not result in a greater perception of dehydration-stress than cells deficient in any one EPS, although they frequently exhibited higher levels of p*PalgD-gfp* expression per cell and for longer periods of time (data not shown). The proportion of wild type and EPS-deficient mutant cells expressing the constitutive control p*PnptII-gfp* reporter was the same under water-limiting and -replete conditions (data not shown).

Table 6. Proportion of 12 h old *P. putida* biofilm cells expressing the *algD* promoter-*gfp* transcriptional fusion during growth under water-limiting conditions [a].

Strain	% GFP expressing cells[b]	
mt2 (pP*algD-gfp*)	82.5	A[c]
Δ*peaGHI* (pP*algD-gfp*)	87.9	B
Δ*bcsQAB* (pP*algD-gfp*)	88.0	B
Δ*bcs*Δ*pea* (pP*algD-gfp*)	88.1	B
Δ*bcs*Δ*alg* (pP*algD-gfp*)	88.5	B
Δ*pea*Δ*alg* (pP*algD-gfp*)	88.7	B
Δ*algD* (pP*algD-gfp*)	92.0	B

[a] The water potential of the medium was lowered by 1.5 MPa with PEG amendments.
[b] The flow cytometer was gated such that *gfp*-expressing cells have a fluorescence intensity greater than 99% of a cell population cultivated under water-replete conditions. Data are the representative mean of one of four comparable experiments each comprised of three replications with similar results.
[c] Values followed by the same letter are significantly similar to one another (P≥0.05, LSD)

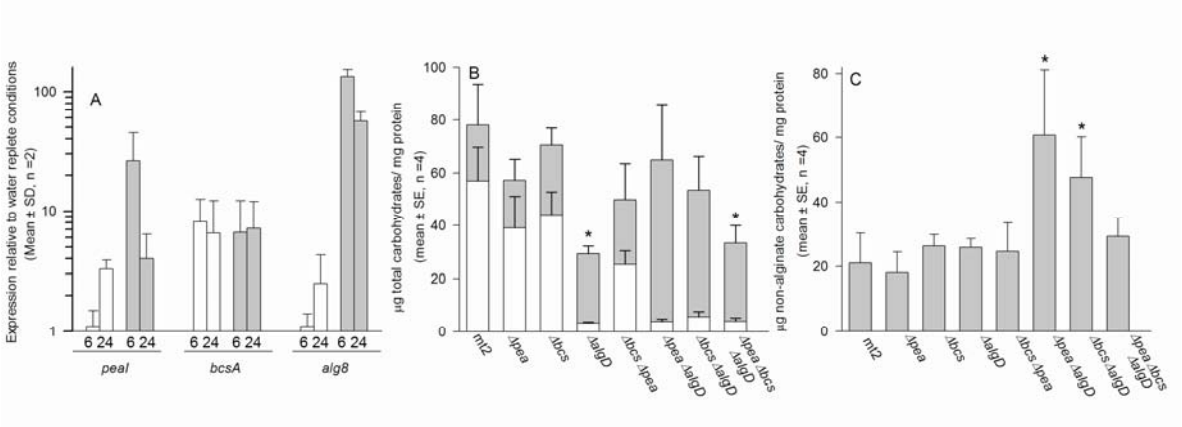

Figure 4. Influence of water-deprivation on EPS gene expression and EPS production by mt2 and EPS-deficient mutants. (A) Effects of a 1.5 MPa reduction in water potential imposed by NaCl (white bars) and PEG (gray bars) on EPS gene expression by 6 or 24 h old mt2 biofilms as measured by qRT-PCR. (B) Alginate (white bars) and total high molecular weight EPS (gray bars) recovered from 24 h old biofilms cultivated on media in which the water-potential was lowered by 2.5 MPa with PEG amendments. (C) Non-alginate proportion of total EPS. Asterisks indicate total (A; P<0.06) and non-alginate (B; P<0.05) carbohydrate yields are significantly different from the wild type, based on an LSD.

EPS production by Bcs and Pea mutants

Our observation of increased *peaI* and *bcsA* expression in mt2 biofilms and increased alginate reporter expression by Δ*bcsQAB* and Δ*peaGHI* under water-limiting conditions lead

us to quantify the alginate (uronic acid) and total carbohydrate contents of ethanol-precipitated high-MW EPS material isolated from biofilms. Under water-replete conditions, there was no statistically significant difference in the total and uronic acid (alginate) contents recovered from 24 h old biofilms formed by mt2 and the EPS-deficient mutants (data not shown). Although there was a slight increase in alginate reporter expression in the Δ*bcsQAB* and Δ*peaGHI* mutants at 12 h (Table 6) it did not lead to increased alginate production in 24 h old biofilms (Fig. 4B). Significantly more non-alginate carbohydrates were recovered from biofilms formed by Δ*bcs*Δ*algD* and Δ*pea*Δ*algD* than from mt2 or Δ*bcsQAB,* Δ*peaGHI*, Δ*algD,* Δ*bcs*Δ*pea,* and Δ*bcs*Δ*pea*Δ*alg* (Fig. 4C). As a consequence, only Δ*algD* and Δ*bcs*Δ*pea*Δ*alg* produced significantly lower amounts of total EPS material compared to mt2 (Fig. 4B). Taken together, our findings indicate that alginate is the primary EPS used to facilitate matrix hydration under desiccating conditions but in its absence cells can increase Bcs and Pea production presumably to maintain matrix hydration necessary for cell survival.

Discussion

Bacteria in biofilms are surrounded by an extracellular matrix which functions to facilitate biofilm development and to provide structure and stability to mature communities. We provide evidence that two gene clusters, one encoding a cellulose-like polysaccharide (Bcs) and putida exopolysaccharide A (Pea) play important roles in cell-surface and cell-cell interactions necessary for biofilm formation under fully hydrated conditions. Furthermore, we show that Bcs contributes to rhizosphere fitness and that both Pea and Bcs may contribute to facilitating matrix hydration under desiccating conditions.

We defined the *pea* locus to be comprised of 9 genes in one predicted operon and 5 additional transcriptional units. All 17 predicted gene products have amino acid sequence similarities with proteins known to function in carbohydrate processing, polysaccharide synthesis, or polysaccharide transport. Based on sequence similarities to enzymes with known substrates we postulated that the putative Pea polysaccharide might consist of

glucose, mannose, and rhamnose residues and our carbohydrate analyses of purified *pea*-dependent matrix material were consistent with this hypothesis. Putida exopolysaccharide A is structurally distinct from the glucose, mannose, and rhamnose containing *P. aeruginosa* exopolysaccharide Psl (Byrd et al., 2009) given its lack of cross-reactivity to Psl-antisera (Appendix A). Based on congo red and calcofluor-staining, and cellulose-sensitivity, Pea contains β-1,3 and/or β-1,4 linkages. The Δ*peaGHI* mutants were not impaired in the initial (6 h) stages of biofilm formation but did exhibit profound biofilm deficiency post-attachment (Figs. 1 and 2, Table 3) under conditions in which the medium osmolarity (~-1.1 MPa water potential) was similar to that used to explore the effect of high osmolarity on *peaI* transcription (Fig. 4A). The inability of Δ*peaGHI* to maintain pellicles (Fig. 1) or to develop robust biofilms over time (Fig. 2 and Table 3) and their resistance to cellulase-mediated inhibition of biofilm development (Table 4) suggest that Pea is central to maintaining cell-cell interactions post-attachment, possibly by maintaining matrix stability.

Based on sequence analyses, all 10 predicted gene products of the *bcs* operon have amino acid sequence similarities to enzymes known to function in cellulose synthesis, processing, or transport in a diverse group of enteric bacteria (Table 1) or are associated with cellulose synthesis operons. Although we do not directly address whether cellulose is made, the similarity of the gene products to known cellulose biosynthetic machinery and that robust calcofluor staining is *bcs*-dependent (Fig. 2C), strongly suggests that cellulose is synthesized. The inability of Δ*bcsQAB* to maintain biofilm biomass in gently washed microtiter plate wells (Fig. 2) suggest that Bcs is involved in maintaining biofilm structure post-attachment, possibly by reinforcing Pea-mediated cell-cell interactions. Additionally, Δ*bcsQAB* is reduced in rhizosphere colonization ability during competition with wild type cells suggesting that Bcs is important for maintaining biofilm structure post-attachment on plant roots, as observed with other cellulose producing plant-associated bacteria (Gal *et al.*, 2003, Matthysse *et al.*, 2005, Matthysse *et al.*, 2008).

Our composition analysis of the EPS obtained from strain mt2 is in congruence with the composition reported previously (Priester *et al.*, 2006). We also observed that the EPS

and CPS compositions are similar to each other, supporting the idea that EPS is cell-bound during growth but is sloughed off when nutrients become limiting (Kachlany *et al.*, 2001). Putida exopolysaccharide A may also be tightly associated with the cell like Psl (Ma *et al.*, 2009) given that we isolated substantially less CPS from biofilms formed by Δ*peaGHI* than mt2 or Δ*bcsQAB* (data not shown).

We were intrigued by our observation that biofilm surface area coverage at the air-liquid interface of glass cover slips by Δ*bcs*Δ*pea* was equivalent to the wild type rather than like Δ*peaGHI* (Table 3 and 4) and that complementation with pME-Bcs (Fig. 3; Appendix A) resulted in partial restoration towards the decreased biofilm formation phenotype of Δ*peaGHI*. Collectively, these data suggest that Bcs interacts with an as yet identified surface adhesin, such as, LapA, curli fibrils, or another EPS. Our data indicate Pea, but not Bcs, is the primary cellulase-sensitive component, which could be the cellulase-sensitive component interacting with the LapA adhesion that dissociates from the cell to facilitate starvation-induced cell dispersion (Gjermansen *et al.*, 2010); it may also interact with the recently described LapF adhesion involved in biofilm maintenance post-attachment (MartÌnez-Gil *et al.*, 2010). Gualdi et al (2008) reported that cellulose modulates *E. coli* biofilm formation by counteracting curli-mediated adherence to solid surfaces directly rather than by negative regulation of curli synthesis. This is based on the observation that cellulose- but not curli-deficient mutants exhibit enhanced biofilm formation (Gualdi *et al.*, 2008). Since the *P. putida* genome sequence (Nelson et al., 2002) revealed the presence of curli biosynthesis genes it is conceivable that the cellulose-like polymer interacts with these amyloid fibrils to optimize cell-surface interactions. In *Streptomyces coelicolor*, evidence suggests that interactions between amyloid and cellulose fibrils facilitate matrix stability and cell-cell interactions (De Jong et al., 2009). Similarly, the agglutinin CdrA was shown to interact with the *P. aeruginosa* exopolysaccharide Psl, presumably to reinforce the biofilm matrix (Borlee et al., 2010). Thus, emerging evidence indicates the biofilm matrix is much more complex and ordered than previously suspected and that individual EPS components can be stabilized by or associates with protein components.

In addition to mediating attachment or facilitating cell-cell interactions during biofilm development, exopolysaccharides are also implicated in providing protection from environmental stresses. The ability of EPS to confer protection from stress is presumably dependent on polysaccharide composition. In this report, we build on our previous studies showing that alginate production by *P. putida* under water-limiting conditions influences matrix physiochemical properties and cell fitness (Chang et al., 2007) to provide several lines of evidence indicating that other EPS constituents may also play roles in desiccation tolerance. The dramatic transient increase in *pea* expression under water-limiting conditions paralleled the pattern observed for alginate while expression of the *bcs* operon increased equally under water-limiting and high osmolarity conditions suggesting it is regulated differently than Pea and/or alginate (Fig. 4A). At present it is unclear whether increased *pea* and *bcs* expression leads to increased Pea or Bcs synthesis since their production and/or secretion may be allosterically regulated by c-di-GMP at the post-translational level. Future work will need to determine whether AlgU, RpoS, or other unidentified regulators control expression of Pea and Bcs.

Cells deficient in making one or more EPS experienced greater dehydration-mediated cell-envelope stress, leading to increased alginate promoter activity compared to the wild type (Table 4). Although the levels of stress severity differ, our quantitative analysis of EPS production showed that increased alginate promoter activity did not lead to increased alginate production (Fig. 4B). This could be a consequence of allosteric regulation of alginate export by c-di-GMP (Merighi *et al.*, 2007) or because a sufficient amount of alginate is present to keep the cells fully hydrated.

It is intriguing to speculate that Pea and Bcs may facilitate matrix hydration in the absence of alginate production since, unlike under water-replete conditions, we recovered more non-alginate carbohydrates in EPS isolated from biofilms formed by Δ*bcs*Δ*alg* and Δ*pea*Δ*alg* than by Δ*bcs*Δ*pea*Δ*alg* (Fig. 4C). This suggests that when unable to synthesize alginate, *P. putida* compensates by producing more Pea or Bcs, presumably to facilitate biofilm hydration. At present it is unclear whether increased EPS production by Δ*bcs*Δ*alg*

and Δ*pea*Δ*alg* is a consequence of disruptions in the normal cell-cell or cell-surface interactions required for creating a hydrated microenvironment or normal water-retention properties of the matrix. Several studies have previously shown that cellulose production contributes to desiccation tolerance and long-term persistence in a desiccated state (White *et al.*, 2006, Gualdi et al., 2008). Our findings lead us to hypothesize that both Pea and Bcs would contribute to desiccation tolerance when fully hydrated biofilms are exposed to a drying event particularly under conditions where alginate production is limited, such as when available nutrients are insufficient to meet metabolic requirements for alginate production. Additionally, we would anticipate that when biofilms form under water-limiting conditions cells would rely on alginate production to facilitate matrix hydration. We are presently exploring how interactions between alginate, Pea, and Bcs influence desiccation tolerance and the ecological success in water-limiting habitats.

Several lines of evidence lead us to conclude that *P. putida* produces at least one additional unidentified exopolysaccharide, such as putida exopolysaccharide B that is described by Nilsson et al (2010) in the accompanying article. First, we observed that older Δ*peaGHI* colonies exhibit a congo red staining phenotype (Fig. 1) and second, we recovered equivalent amounts of non-alginate carbohydrates from Δ*bcs*Δ*pea*Δ*alg* and the wild type under water-replete and -limiting conditions; we anticipated the wild type would produce more EPS than the triple mutant. Nilsson et al (2010) used *P. putida* KT2440 as a model organism, which is a restriction-negative derivative of strain mt2 devoid of the conjugative plasmid pWWO; use of different strains may explain why our results differ in some points with theirs.

Our data indicate a previously uncharacterized locus, *pea*, may encode a novel polysaccharide that is central for maintaining cell-cell interactions necessary for biofilm matrix stability. We also show that the bacterial cellulose operon encodes a polymer that contributes slightly to biofilm matrix stability. Future experiments will include a thorough investigation into the structure of each polymer, and their potential interactions with protein components of the biofilm matrix and how those interactions influence biofilm

formation, matrix stability, and matrix physiochemical properties. Collectively, the data suggests that Pea, Bcs, and alginate contribute to fitness under water-limiting conditions, but alginate may have a particularly important role.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Strains, plasmids, and PCR primers used in this study are listed in Tables 2 and Appendix A. *E. coli* was grown at 37 °C while *P. putida* was grown at 28 °C. Jensen's medium (JM) (Jensen *et al.*, 1980, Jackson et al., 2004) with 20 mM mannose or TYE (Li et al., 2010) were prepared as described previously. To simulate a -1.5 MPa matric water stress media was amended with polyethylene glycol 8000 (PEG), as previously described (van de Mortel and Halverson, 2004). For flow cytometry experiments we used ¼- strength TYE containing 1.25 g of tryptone, 0.67 g of yeast extract, 0.7 g KH_2PO_4 and 40 ml of Hunter's mineral solution (Smibert and Krieg, 1994) per liter. If required, antibiotics were added at the following concentrations: kanamycin (Km) and ampicillin (Ap) 50 μg mL^{-1}, tetracycline (Tc) 15 μg mL^{-1}. Plates were incubated in closed plastic containers to maintain the desired relative humidity.

DNA manipulation and mutant construction

Plasmid and genomic DNA was extracted according to manufacturer's protocol using the Qiagen mini-prep and Wizard Genomic DNA kit (Promega), respectively. DNA concentrations were measured using a Thermo Scientific Nanodrop ND-1000 and cloned inserts were confirmed by sequencing analysis. If needed, SacI, XbaI, or EcoRV (NEBiolabs) restriction enzymes were used to liberate inserts and ligated (T4 ligase, Promega) into similarly digested plasmids. PCR amplifications used Ex-Taq (Takara) or Phusion Taq (Finnezymes) using an MJ-1000 thermocycler. Construction of the *algD* deletion was described previously (Chang et al., 2009). Construction of all unmarked deletions of *peaGHI* (PP3139-3141) and *bcsQAB* (PP2634-2636) were as follows. First, 800-1000 bp fragments flanking genes targeted for deletion were amplified using PCR primer pair

PP3138F/Km3138Up, and Km3141Dn/PP3143R2 for Δ*peaGHI,* and 2632SOE1F/ 2633SOE2R and 2636SOE5F/2637SOE6R for Δ*bcsQAB* (Appendix A). The FRT-Km resistance cassette was PCR amplified from pKD4 (Hoang et al., 1998) with 20-30 bases of sequence overlapping the upstream or downstream PCR products flanking *peaGHI* and *bcsQAB* genes. A second round of PCR amplification using splice overlap extension procedures (Yu *et al.*, 2000, Choi *et al.*, 2005) created pea-FRTkm and bcs-FRTKm, which were then cloned into pGemT-Easy to create pGpea-FRTKm and pGbcs-FRTKm. Cloned PCR products were removed by restriction digestion, gel purified, and ligated into pEX18Tc to generate pEX-pea and pEX-bcs which were then transformed into *E. coli* TOP10 cells prior to isolation and electroporation into *P. putida*. Deletion mutants were obtained by sequentially screening for Km^R and Tc^S colonies in the presence of 5-10% sucrose and verified by PCR using external primers (Appendix A and Figure 4S). Unmarked deletions were constructed by introducing pFlp2 (Hoang et al., 1998) to excise the FRT-Km cassette. To create pyramided deletion mutants, plasmid pEX-pea was electroporated into Δ*bcsQAB* and pEx-Δ*algD* (Chang et al., 2009) into Δ*bcsQAB,* Δ*peaGHI,* and Δ*bcs*Δ*pea*. Mutants were verified using PCR to verify reduced sizes of genomic regions (Appendix A).

Congo red and pellicle assays

Five µl aliquots of overnight TYE-grown cultures were spotted onto LB plates containing 40 µg/mL congo red and 15 µg/mL coomassie blue. Red or pink colonies on congo red plates indicated the binding of congo red to extracellular matrix material. For pellicle assays 10 µl aliquots of 100-fold dilutions of overnight TYE-grown cultures were inoculated in 20 mL of TYE media containing 0.3 M NaCl and incubated statically for 1-7 days.

Coverslip biofilm assay and cellulase treatment

P. putida strains were chromosomally tagged with miniTn7gfp_{AAV} in the neutral att site, as described previously (Lambertson et al 2004). Sterile glass coverslips (25x 55 mm) were placed in 50 mL Falcon tubes containing 10 mL of JM medium with or without 200 U

cellulase (Worthington) prior to inoculating with 5 µL of overnight cultures. Tubes were incubated upright at 28 °C on an orbital shaker (50 rpm). Before microscopy the coverslips were removed and washed gently three times with sterile water in separate beakers. Epifluoresence microscopy images were obtained using a Nikon Eclipse 80i system equipped with a Nikon EZ Coolsnap camera using the 10X objective lens and a GFP-HYQ (EX: 450-490nm, DM: 495nm, BA: 500-550nm) filter cube. Comparisons between images was done after standardization of images to a 12-bit luminescence range, background subtracting non-colonized areas by excluding pixels with luminescence values below that of *gfp*-expressing cells, and counting the remaining pixels using the 'region measurements' tool of MetaVue v7.1 software (Molecular Devices). Data was transferred into an Excel spreadsheet to calculate the proportion of surface area coverage by dividing the number of pixels with luminescence values greater than background by the total number of pixels. A one-way ANOVA was performed using JMP 8.0 by pooling experiments comprised of three replicates per experiment.

Calcofluor staining

The coverslip biofilm formation assay was performed as described above with the following alterations: cells were not miniTn7gfp_{AAV} tagged, coverslips were dipped into 100 µM calcofluor solution for 10 seconds, and washed twice. Coverslips were visualized by epifluorescent microscopy using the UV-2E/C (Ex: 325-375nm, DM: 400nm, BA: 435-485nm) filter cube. Ten-bit images were captured and processed as described above to remove non-specific background fluorescence. To compare the calcofluor staining by the mutants, images were analyzed by creating a histogram of pixel fluorescent intensities using the 'intensity profile' display option within MetaVue.

Microtiter dish biofilm assay

Assays were performed essentially as described by O'Toole & Kolter, 1998. Briefly, overnight cultures were diluted 1:10 into fresh TYE medium and 10 µL were inoculated into PVC Falcon 3911 microtiter plate wells containing 100 µL TYE and incubated statically. For 6

h assays, dense overnight cultures were diluted 10-fold in fresh TYE medium and inoculated directly into the microtiter plate wells. At each time point, non-attached and loosely adhering cells were removed and the wells were rinsed by gently submerging the plate in water 3 times prior to air-drying. Next, 125 µl of a 0.4 % crystal violet solution was added to each well and the plate incubated at room temperature for 15 minutes. The washing process was repeated. Bound crystal violet staining the cells was solubilized with 100 % ethanol and a 100 µl aliquot was transferred to a new microtiter plate and the absorbance read at 545 nm using a Biotek EL 340 microtiter plate reader.

Complementation of Pea and Bcs

PeaGHI and *bcsQAB* genes were PCR amplified using primer sets CompPea-F/CompPea-R2 and BcsCompF/BcsCompR. PCR products were blunt end ligated into EcoRV-digested pME6041 to create pMe-Pea and pMe-Bcs and transformed into *E. coli* TOP10 cells. pMe-Pea and pMe-Bcs were isolated and electroporated into cells to create Δ*peaGHI* (pMe-Pea), Δ*bcsQAB* (pMe-Bcs), and Δ*bcs*Δ*pea* (pMe-Bcs). Complementation strains and their pME6041 empty vector controls were assayed for biofilm formation using the coverslip biofilm assay described above in the absence of cellulase.

Seed attachment and competitive root colonization assays

Attachment to *Zea mays* Mo17 corn seeds followed previously published methods (Espinosa-Urgel *et al.*, 2000). Briefly, stationary phase cultures were diluted 1:1000 into fresh M9 medium and enumerated by plate counts. Seeds were surface sterilized, and hydrated in sterile water for 12-16 h prior to transferring to a tube containing 1.0 mL of the diluted bacterial suspension and incubated at room temperature for one hour. Seeds were then removed, washed gently with water to remove loosely adhered bacteria, transferred to a borosilicate test tube containing glass beads and 1.0 mL fresh M9 medium, and vortexed before enumerating cells by plate counts. Two corn seed attachment assays were performed each comprised of 3 replicates and 3 sub-samples per replicate. A one-way ANOVA was performed using JMP 8.0.

For competitive root colonization assays, corn seeds were surface sterilized and germinated in the dark for 48 h on dampened, sterile filter paper and then planted in 50 mL Falcon tubes containing sterile sand (Yousef Coronado *et al.*, 2008). A mixture of LB-grown stationary phase mt2 (Km^S) and either Δ*peaGHI*-Km, Δ*bcsQAB*-Km, or Δ*bcsQAB*Δ*peaGHI*-Km (Km^R) cultures were mixed in a 1:1 (wild type: EPS mutant) ratio in plant nutrient solution (Ramos-Gonzalez et al., 2005) and enumerated on TSA and TSA+Km. Ten mL of mixed bacterial solutions were added to the Falcon tubes containing planted corn and loosely covered with plastic wrap and incubated at 28 °C, 55-65 % relative humidity with a daylight period of 16 h for 7 days. Plants were removed from the soil, gently rinsed with sterile water, and roots separated from the plant. Roots were weighed prior to placing in 20 mL of M9 medium containing glass beads. Samples were vortexed for 2 minutes to remove attached cells and the suspensions plated onto TSA and TSA+Km to enumerate cells. Three separate experiments were performed each comprising of 3-12 replicates. Significant changes between the proportion of initial and final EPS-deficient populations were assessed using Tukey-Kramer and contrast analysis by SAS v9.0 software.

Quantitative real-time PCR

P. putida was cultivated overnight on Kings B medium, resuspended in ½-strenth 21C medium (Chang et al., 2007) with glucose and succinate to an OD_{600} nm of 0.4 and incubated for 15 min prior to inoculating plates with and without NaCl or PEG to lower the water potential by 1.5 MPa. The 6- and 24-old biofilms were harvested in the presence of RNA Protect (Qiagen) prior to RNA extraction and DNase treatment as described previously (Li et al., 2010). RNA was quantified with a Nanodrop-1000 and integrity verified using a Bioanalyzer (Agilent). Primers (Appendix A), QScript 1-step SYBR kit (VWR), and the iCycler (BioRad) thermocycler was used for amplification and quantification. Data was normalized to *rimM* and expressed relative to water-replete controls using the ΔΔCt method (Livak and Schmittgen, 2001).

EPS/CPS isolation, quantification, and composition analysis

EPS was isolated from 24 or 48 h-old biofilms grown on a nylon membrane overlaying water-replete (basal) or PEG amended TYE medium to lower the water potential by 1.5 MPa before removing membranes and suspending cells in 0.85 % saline solution. An aliquot was removed for protein and cell enumeration and the remaining cells centrifuged to separate the EPS-containing saline solution from the cells. For CPS isolation, the resulting cell pellet was suspended in 1 mM phosphate buffered saline and the CPS was sheared off the cells using a Waring blender prior to centrifugation (Kachlany et al., 2001). The CPS and EPS-containing supernatants were dialyzed (3,500 MWCO dialysis cassettes, Thermo) against water for two days. High molecular weight CPS and EPS was precipitated with 3X volumes of ice-cold ethanol at -20 °C for 1-3 days and then centrifuged at 16,300x g. The pellet was air dried and resuspended in water. Total carbohydrates were determined by the phenol-sulfuric acid method with glucose as a standard, and uronic acids were determined by the *meta*-hydroxydiphenyl-based method using D-glucuronic acid as a standard (Blumenkrantz, 1973, Fox and Robyt, 1991). Carbohydrate content was normalized to biofilm biomass as determined by the Bradford protein assay. We performed ANOVA on $\log_{10}$ transformed total carbohydrate and uronic acid yields and means were compared by LSD using JMP 8.0.

Glycosyl residues were identified using 150 µg EPS or CPS after derivatization and GC/MS analysis according to a modified protocol of York *et. al.* 1986. Briefly, sugars were heated in methanolic HCl, re-N-acetylated with pyrindine and acetic acid, and then per-O-trimethylsilylated with Tri-Sil (Pierce) (Merkle and Poppe, 1994). GC/MS analysis was done using a 30m x 0.25 mm ID DB-5 column in a HP6890/5793 GC-MS with the following program: initial temperature of 140 °C for 2 minutes; ramp temperature 0.5 °C per minute; final temperature 275 °C. Myo-inositol was used as an internal standard (York *et al.*, 1986). Glycosyl residues were identified using the mass spectrometry library and by manual inspection of the chromatograms. Sugars were expressed as percentage of total carbohydrates.

Crude EPS extraction and westen immunoblotting using anti-Psl serum

Crude EPS extracts were obtained from 5 mL of late stationary cells grown in JM plus mannose broth and concentrated to 10 OD units. Cell pellets were resuspended in 100 µL 0.5M EDTA, boiled for 5 min and centrifuged. Proteinase K was added to the supernatant to a final concentration of 0.5 mg/mL and incubated at 60 °C for 1 h prior to inactivation at 80 °C for 30 min (Byrd et al., 2009). Crude EPS samples were spotted onto nitrocellulose membranes and air dried. Immunoassays were performed essentially as described by Byrd *et. al* 2009 with the modification that we diluted (1:12500) α-Psl IgG serum in TBST and exposed the membrane for 2 rather than 1 h. After removal of primary antibody and washing, the blot was incubated with 1:10000 Donkey α-rabbit IgG-HRP in TBST (Southern Biotech) for 1 hour. HRP activity was detected using HyGlo chemiluminescent HRP antibody detection kit (Denville Scientific) and visualized using a Kodac 440CF gel doc station.

Alginate biosensor and pPnptII-gfp reporter assay

p*algD-gfp* and *pPnptII-gfp* were electroporated into *P. putida* strains. Cells were grown for 18 h on ¼ TYE plates prior to suspension and dilution in ¼ TYE broth to an OD_{600} of 0.1 and further diluted 100 to 1000-fold prior to inoculating multiple 5 µl aliquots onto ¼ TYE plate with or without PEG amendments to lower the water potential by -1.5MPa. After 12 h, biofilms were either scraped from the inoculated areas into low salt buffer. If needed, Syto 60 red fluorescent dye was used to ensure all counted particles were cells during flow cytometry analysis. Flow cytometry was performed at the Iowa State University Cell Facility using a BD FACSCanto flow cytometer equipped with 488 nm excitation argon laser and 530 nm or 610 nm emission filters for GFP and Syto 60, respectively. Gates were set based on the GFP fluorescence intensity being greater than 99% of the similar cell population grown under water-replete conditions.

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Chapter 4. The multifaceted interactions between a novel, secreted amyloid-like protein and exopolysaccharides of *P. putida* on biofilm formation and cell physiology

Summary

The *Pseudomonas putida* biofilm exopolymeric matrix is comprised of multiple exopolysaccharides (EPS), DNA, and proteins. Here, we describe the role of two gene clusters that produce a novel amyloid-like protein that interacts with various EPS components to influence biofilm phenotypic properties and alginate gene expression. Although the original annotation of the *P. putida* strain KT2440 genome indicated gene PP3399 was degenerate we show that it is expressed and produces a secreted formic acid-resistant protein capable of binding the amyloid-indicating dye Thioflavin T and an amyloid conformation-specific antibody, WO1, in wild-type, but not PP3399 mutant cells. We have named the PP3399 gene *psgA* for purlin subunit gene A and its encoded protein, purlin, since it is homologous to the *E.coli csgA* gene and curlin. Purlin contains 481 amino acids and is predicted to form a rod-shaped protein harboring a cross beta-structure motif comprised of three R1 and nine R2 repeat domains connected by a short linker. Purli contributes to the initiation of pellicle formation and biofilm adherence and is most noTable in Pea EPS-deficient cells. *PsgA* transcription increases during growth under water-limiting conditions relative to high osmolarity or water-replete conditions in a manner similar to the alginate biosynthetic operon. All strains, except Δ*bcs*Δ*pea*Δ*psgA*, are mucoid during growth in water-limited conditions suggesting a complex interaction exists between the Pea and Bcs exopolysaccharides, purlin, and alginate production. Biofilm Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* cells have high alginate biosynthetic gene expression in water-replete conditions compared to wild-type and all other EPS and *psg* mutants. Growth in water limited conditions causes at least a 25-fold increase in alginate gene expression compared to water-replete conditions, however, only a marginal fold increase occurred in *ΔbcsΔpsgA* and Δ*bcs*Δ*pea*Δ*psgA* mutants due to high background alginate expression. Together, our

data suggests a complex network of EPS and purli interactions contributes to biofilm formation and fitness under water-limiting conditions.

Introduction

In Chapter 3 we described how the Pea exopolysaccharide (EPS) produced by *P. putida* mt2 is responsible for stable biofilm formation on glass coverslips, yet cells that were unable to produce both cellulase-sensitive EPS polymers, Pea and Bcs, formed similar biofilms as wild-type cells. One possible explanation of this comes from the *E. coli* literature in which cellulose and amyloid-like fiber (ALF), known as curli, work together in a compensatory manner to aid surface attachment and biofilm stability (Gualdi *et al.*, 2008). Two equally plausible models for this have been proposed. The first model suggests that in the absence of a particular EPS, pre-existing adhesins are unmasked for cell-surface and cell-cell attachment, as well as biofilm stabilization (Schembri *et al.*, 2004). In the second model, cellulose and curli are co-regulated and the inability to produce cellulose stimulates gene expression and downstream synthesis of adhesins (Gualdi *et al.*, 2008, Grantcharova *et al.*, 2010). *P. putida* have both cellulose and curli gene homologs, thus, either model may be applicable and explain our previous results. To delineate, which, if either, model is operational in *P. putida,* a thorough understanding of recent findings is necessary.

Adhesins can be of two broad forms: those that function as individual monomer proteins, such as LapA, or those that can self-assemble into multimeric units such as pili, fimbriae, tafi, chaplins, TasA and curli (Espinosa-Urgel *et al.*, 2000, Prigent-Combaret *et al.*, 2000, Chapman *et al.*, 2002, Hinsa *et al.*, 2003, Bokranz *et al.*, 2005, White *et al.*, 2006, De Jong *et al.*, 2009, Romero *et al.*, 2010). Amyloidogenic proteins are defined based on their cross beta-structure and ability to alter the fluorescence intensity of certain fluorogenic dyes, such as thioflavin T, although the requirement for the tertiary structure is debatable (Fändrich, 2007, Shewmaker *et al.*, 2009) Amyloidogenic protein monomers self-assemble into long polymers based on structural and amino acid similarities between each amyloid monomer. In the case of *E. coli* the monomer is known as curlin, while the entire polymeric amyloid fiber structure is called curli. Amyloid fibers can extend into the extracellular space

up to 30 μm and reinforce the biofilm structure suggesting that, akin to exopolysaccharides, ALF can serve as a structural network to connect distantly separated cells and scaffold to support the biofilm matrix (Prigent-Combaret *et al.*, 2000, Romero *et al.*, 2010). The role of ALF in biofilm structure may be common since multiple environmental biofilms bind the amyloid conformation-specific antibody, WO1 (Kikuchi *et al.*, 2005, Larsen *et al.*, 2007). Interestingly, bacterial amyloidogenic proteins are sometimes co-regulated or isolated as a contaminating protein with cellulose (Claessen *et al.*, 2003, Zogaj *et al.*, 2003, Khurana *et al.*, 2005, Xu *et al.*, 2008). This is best characterized in *E. coli* and *Salmonella* species.

E. coli cellulose and curli production are regulated by the CsgD transcriptional regulatory protein (Brombacher *et al.*, 2006). CsgD expression is influenced by various global regulatory systems, such as RpoS, H-NS, FisR, and the EnvZ-OmpR two-component system, and the housekeeping sigma factor, σ^{70} (Olsén *et al.*, 1993, Arnqvist *et al.*, 1994, Vidal *et al.*, 1998, Saldaña *et al.*, 2009). In turn, CsgD directly regulates expression of the *csgBA* and *csgDEFG* operons that encode the curlin subunit proteins, CsgD itself, and other secretory and gating proteins necessary for curli synthesis. CsgD is also indirectly responsible for cellulose production by regulating the transcription of AdrA, a GGDEF-domain protein producing the second messenger molecule cyclic-di-GMP that allosterically activates cellulose synthase (Arnqvist *et al.*, 1994). Overall, CsgD over expression leads to increased biofilm formation in *E. coli* Δ*bcsA* and Δ*csgA* mutants but not in Δ*bcsA*Δ*csgA* cells suggesting a compensatory effect of Bcs and CsgA on biofilm properties (Pamp *et al.*, 2009, Saldaña *et al.*, 2009). However, in most cases, it is unknown if cellulose and ALFs directly interact with one another (White *et al.*, 2006, Saldaña *et al.*, 2009).

A recently proposed model suggests that the *S. coelicolor* amyloid monomer units, ChpA, associate with a cellulase sensitive exopolysaccharide to contribute to surface attachment and structural stability of biofilms (De Jong *et al.*, 2009). Extracellular fibrous structures in chaplin-deficient mutants have reduced thickness compared to wild-type cells and the addition of an ALF-inhibitor decreases *S. coelicolor* attachment to abiotic surfaces (De Jong *et al.*, 2009). Electron microscopy of chaplin-deficient mutants revealed thin,

cellulase-sensitive filaments anchored to the cell surface by a spiked structure. Cellulase treatment of wild-type *S. coelicolor* cells completely abolished the presence of filamentous structures and cell attachment. The authors propose a model in which cellulose-like polymers serve as an aggregation point for chaplin monomers and, together, are anchored to the cell surface by the spiked structures. This model is similar to another model in that cellulose is tightly associated with thin aggregative fimbriae in *S. enterica,* although a direct cellulose-fimbriae interaction has not been demonstrated (White *et al.*, 2006). Amyloidogenic fibers are not the only extracellular proteins that interact with EPS constituents to promote biofilm matrix stability since CdrA, a long, β-barrel rich, hemagglutinin protein produced by *P. aeruginosa* was recently shown to associate with Psl EPS and provide biofilm structural stability (Borlee *et al.*, 2010). *P. putida* biofilms are stabilized by interactions between LapA and a cellulase-sensitive EPS (Gjermansen *et al.*, 2010).

In this study we describe the novel purlin subunit gene A, *psgA*, in *P. putida* and test if purli shares common characteristics of other known amyloid-like fibers. We then explore if purli is responsible for reversion of Δ*bcs*Δ*pea* cells back to wild-type levels of biofilm surface area coverage on glass coverslips and whether it interacts with Pea to influence biofilm properties. We then test if the level of *psgA* gene expression is altered by growth in water-limited habitats since biofilm cells produce more EPS during matric conditions than high osmolarity or water-replete conditions, implying that the biofilm matrix would need greater structural support. Lastly, we expand on our observation that PsgA deficiency leads to a non-mucoidy phenotype in bcs mutants that coincides with disrupted alginate gene expression under both water-replete and water-limited conditions.

Results

Purli operon structure

An analysis of the *P. putida* KT2440 genome sequence revealed two gene clusters with one or more genes homologous to the best-studied amyloidogenic appendages, called

curli, that are produced by various strains of *E. coli*. We refer to these genes as the purli genes and adopted the nomenclature of *psg* genes where proteins that have homology to *E. coli csg* operon genes retained the same letter designation (Fig. 1). Although there are some similarities to the *E. coli* curli genes, there are distinct differences, such as the presence of a larger number of genes potentially involved in purli synthesis, the gene clusters are separated by great distance rather than being adjacent to each other, and the presence of at least three putative transcriptional units (Fig. 1A). Notably, we failed to identify a homolog of the *E. coli* CsgD transcriptional regulator in *P. putida* based on BLASTP analysis, as well as the *csgC* gene that, although not involved in curli production, is responsible for the autoaggregative phenotype of *E. coli* (Hammar *et al.*, 1995).

A putative *psgA* promoter region with a predicted σ^{70} binding site 303 bp upstream of the translational start site was identified by the BPROM (www.softberry.com) algorithm. Eight bases upstream of the predicted *psgA* start codon is a putative ribosomal binding site (RBS) whose sequence differs by one nucleotide from the *E. coli* Shine-Delgarno sequence (Fig. 1A). *E. coli csgBA* genes are encoded on a single transcriptional unit and are not thought to contain an internal *csgA* promoter site within the *csgB* gene. Due to this potential novelty, we used reverse transcriptase PCR to confirm our prediction that the psgA promoter was embedded within the *psgB* gene. The results supported the BPROM program prediction that an independent *psgA* promoter site exists within the *psgB* gene (Fig. 1B). The FindTerm program was unable to predict any rho-independent transcriptional after the *psgA* gene and we were unable to assess if there are any rho-dependent terminators.

Computation analysis of PsgA

The PsgA amino acid sequence contains a predicted Sec-signal sequence followed by three R1 domain repeats, a linker region, and nine R2 domain repeats with the consensus sequence QX_4-NX_5-$QX_{1\text{-}3}$-GX_2-NX_4 (Fig. 2A, 2B). These repeats are comprised of highly conserved patterns of hydrophilic, polar amino acids that were identified by the

RADAR program algorithm (Fig. 2B) (Heger and Holm, 2000). Consequently, the predicted tertiary structure of PsgA, which includes the SEC-sequence, assumes a long, cross β-barrel architecture and the R1 repeat domains and the R2 repeat domains are clearly visibly separated by the linker region. This structure is similar to those of other amyloid monomers, including the *E. coli* CsgA*, B. subtilis* TasA, and *S. coelicolor* ChpA (Elliot *et al.*, 2003, Wang *et al.*, 2007, Zhang, 2008, De Jong *et al.*, 2009, Romero *et al.*, 2010). One notable difference is that a single PsgA monomer contains 481 amino acids making it at least three-times the length of the *E. coli* CsgA based on structural predicitions (data not shown).

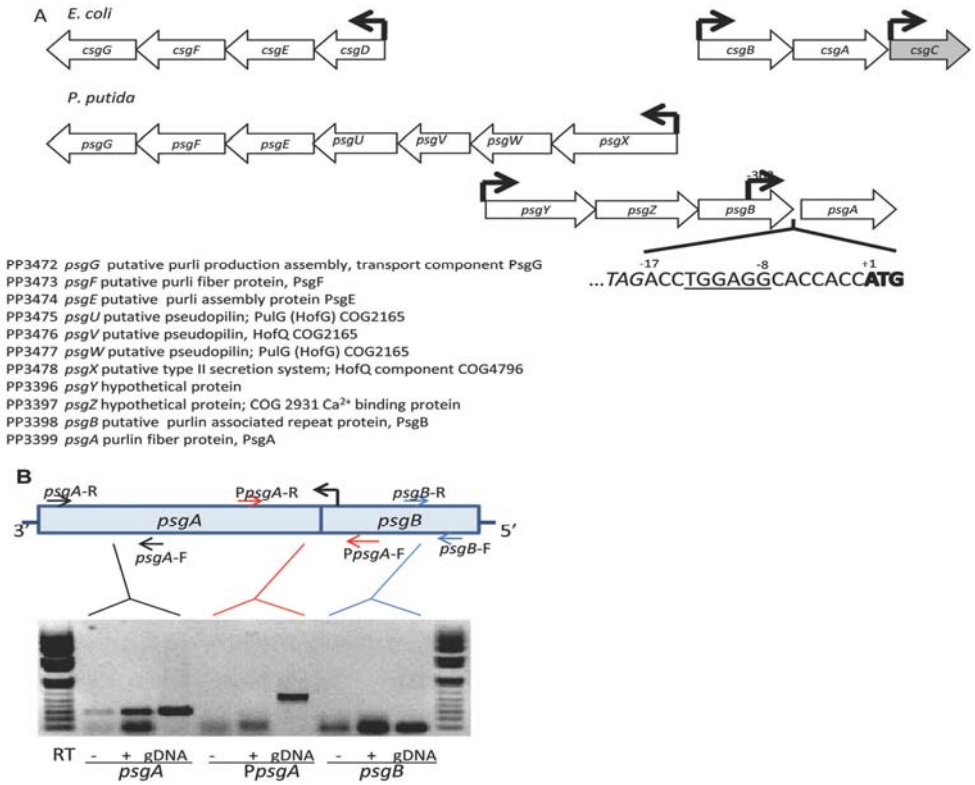

Figure 1. Comparison of *E. coli* curli and *P. putida* purli genes (A) and demonstration that *P. putida psgA* transcript is distinct from the *psgB* transcript (B). (A) Organization of the *P. putida psg* operon structure compared to *E. coli* and predicted function of each *psg* gene. The 17 bp intergenic region between the *psgB* stop codon (italics) and *psgA* translational start site (bold) contains a putative ribosomal binding site (underlined). Numbers listed above the intergenic sequence between the *psgB* and *psgA* genes are base pair positions relative to the +1 *psgA* translational start site. Genes are not drawn to scale. (B) RT-PCR demonstrating that we did not detect a multigenic *psgB* and *A* transcript but we could detect a *psg*A transcript. Genomic DNA (gDNA) or two independently isolated RNA samples (S1, S2) were used as templates for RT-PCR with primer pairs psgA-FPr/psgA-

RPr (blue arrows) and psgAInF/psgAInR (red arrow) either in the presence (+) or absence (-) of reverse transcriptase. NP refers to a genomic DNA sample containing reverse transcriptase but no primers.

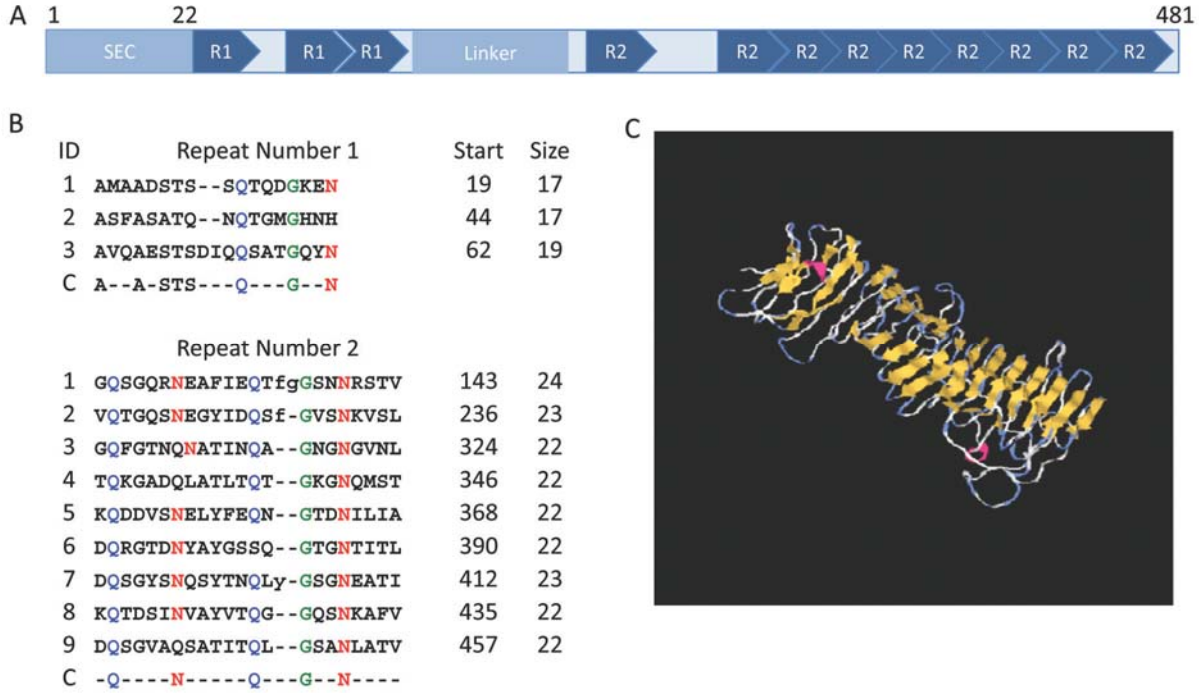

Figure 2. Model of PsgA structure. A) Schematic of the primary structure illustrating the predicted SEC, linker, and repeat R1 and R2 domains. (B) PsgA is comprised of three R1 and nine R2 repeating units and each repeating unit is equivalent to one □-sheet strand predicted by the RADAR program algorithm. The start site and size (number of amino acid residues) of each repeat is indicated (Heger and Holm, 2000). Consensus sequence was manually constructed. (C) Diagram of the tertiary structure model of PsgA as predicted by the iTasser server algorithm (Zhang, 2008).

Strain constructions

In order to perform an analysis of the role of purli in *P. putida* biofilm formation and stress tolerance, we constructed purli-deficient mutants using standard allelic replacement and marker excision procedures. We selected the *psgA* gene for mutagenesis since it appears to constitute a single gene transcriptional unit and it encodes the subunit necessary for creating amyloid-like fibers. Since we were concerned about accumulating secondary mutations in our strains with *psgA* and one or more EPS gene deletions we pyramided *psgA* deletions onto previously constructed EPS-deficient strains and *vice versa* (Table 1).

Partially purified purli fibers bind Thioflavin T

Proteins secreted into the biofilm matrix were isolated by centrifugation of ammonia-sulfate precipitated proteins prior to formic acid treatment. Formic acid disrupts all proteins and depolymerizes amyloidogenic proteins into their monomeric components (Hammar *et al.*, 1996). Following formic

Table 1. Strains and plasmids used in this study

Strain or Plasmid	Characteristics	Source or Reference
Strain		
E. coli MC4100	Wild-type curli producing strain	(Chapman *et al.*, 2002)
Δ*csgA*	MC4100 curli-minus derivative	(Chapman *et al.*, 2002)
P. putida mt2	Wild-type, Rf^R	(van de Mortel and Halverson, 2004)
Δ*psgA*	mt2 *psgA* deletion, Rf^R	This study
Δ*bcs*Δ*psgA*	Δ*bcsA*Δ*psgA* in frame deletions, Rf^R	This study
Δ*pea*Δ*psgA*	Δ*peaGHI* pyramided with Δ*psgA* deletion,	This study
Δ*pea*Δ*psgA*1	Δ*psgA* pyramided with Δ*peaGHI* deletion,	This study
Δ*bcs*Δ*pea*Δ*psgA*	Δ*bcs*Δ*psgA* pyramided with Δ*peaGHI* deletion, Rf^R Km^R	This Study
Δ*bcs*Δ*pea*Δ*psgA1*	Δ*bcs*Δ*pea* pyramided with Δ*psgA*	This study
Δ*bcs*	Δ*bcs* in frame deletion, Rf^R	Chapter 3
Δ*pea*	Δ*peaGHI* in frame deletion, Rf^R Km^R	Chapter 3
Δ*bcs*Δ*pea*	Δ*bcs*Δ*pea* in frame deletion mutant, Rf^R	Chapter 3
Plasmids		
pN	pME6041 based expression vector, Km^R	(Chen *et al.*, 2010)
pN-PsgA	pN with *P. putida psgA* genomic fragment,	This study
pP*algD-gfp*	*algD* operon promoter-*gfp* long-lived transcriptional fusion plasmid, Km^R	(Chang *et al.*, 2007)
mini-Tn7-gfp2	long-lived *gfp* marker, Gm^R	(Lambertsen *et al.*, 2004)
pTok2SacB+*psgA*	pTok2SacB gene used for	This Study
pTok2SacBΔ*psgA*	*psgA* allelic exchange vector, Tc^R	This Study
pEX*pea*	*peaGHI* allelic exchange vector, Tc^R Km^R	Chapter 3
pEX*bcs*	*bcsQAB* allelic exchange vector, Tc^R Km^R	Chapter 3
pEX18-AlgD	*algD* allelic exchange vector , Tc^R Km^R	(Chang *et al.*, 2007)

acid treatment amyloidogenic monomers can naturally reassemble and can react with the conformation-specific antibody WO1 and bind fluorogenic thioflavin T (ThT) dye. We found formic acid treated protein samples from wild-type cells over expressing *psg*A reacted with WO1 whereas strains that did not over express *psgA* or *csgA* do not (Fig. 3A). Additionally,

E. coli anti-CsgA polyclonal serum did not react specifically with formic acid treated proteins from *P. putida* but, as anticipated, *E. coli* MC4100 proteins did (Fig. 3A).

Equivalent amounts of *P. putida* and *E. coli* formic-acid treated proteins increased ThT fluorescence intensity four-times more than a ThT only solution (Fig. 3B). In contrast, *P. putida* Δ*psgA* mutants produced a peak equal in intensity to *E. coli* Δ*csgA,* bovine serum album (BSA), and a solution devoid of proteins (Fig. 3B). Complementation of the Δ*psgA* mutant with pN-PsgA restored the fluorescence intensity of ThT to that of wild-type levels. The strong emission spectrum of the wild type *P. putida* mt2 and *E. coli* MC4100 strains, but not their respective Δ*psgA or* Δ*csgA* mutants, provides strong evidence that the *P. putida psgA* gene encodes a protein with amyloid-like properties.

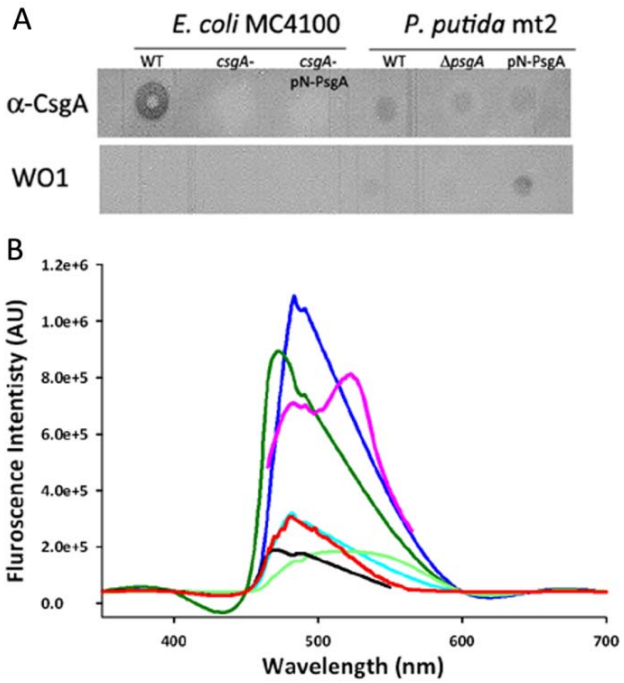

Figure 3. *P. putida* PsgA protein reacts with WO1 antibodies and binds Thioflavin T. (A) Dot blot analysis of formic acid-treated extracellular proteins probed with anti-CsgA or WO1 antibodies. (B) Fluorescence emission spectra of a 20 μM Thioflavin T solution when mixed with formic acid-treated extracellular proteins isolated from *P. putida* mt2 (dark blue), *P. putida* Δ*psgA* (light blue), *P. putida* Δ*psgA* (pNPsgA)(pink), *E. coli* MC4100 (dark green), *E. coli* Δ*csgA* (light green), BSA (red), or ThT only solution (black).

Biofilm Formation

Congo red binding and pellicle formation

The ability of colonies to bind congo red (CR) is one indicator of the composition of the biofilm matrix. Congo red binds both exopolysaccharides and amyloid-like fibers and previous studies have determined that visualization of CR binding by *P. putida* cells is aided by constitutively expressing the cyclic-di-GMP synthesis protein, WspR19, in medium containing NaCl (Chapter 3). For *P. putida* the majority of red pigmentation, indicative of CR binding, is attributed to the Pea EPS. However, this does not entirely alleviate pigmentation since a second congo red binding event occurs. We tested the ability of colonies to bind congo red in various EPS and purli-mutant colonies and observed wild-type, Δ*psgA,* Δ*bcs*, and Δ*bcs*Δ*psgA* colonies become red, as expected since the Pea EPS was present (Fig. 4A). Conversely, Δ*pea,* Δ*pea*Δ*psg,* Δ*bcs*Δ*pea* and Δ*bcs*Δ*pea*Δ*psgA* colonies remained white after 48 h of growth. However, we noticed the formation of numerous petite colonies in the center of the larger colony formed Δ*bcs*Δ*pea*Δ*psgA* but not Δ*bcs*Δ*pea* cells (Appendix C). Interestingly, after 4 days of incubation, some, but not all, of these petite colonies bound CR that could not be attributed to either purli, Pea, or Bcs synthesis (Appendix C). Together this suggests that purli contributes to congo red binding of *P. putida* but is only one of many CR binding materials produced by *P. putida.* This is further supported by figure 4A since Δ*bcs*Δ*pea*Δ*psgA* forms a binds CR around the perimeter of the colony whereas Δ*bcs*Δ*pea* appear the same coloration throughout the entire colony.

Pellicle formation is only possible in *P. putida* cells expressing WspR19. Wild-type and EPS mutant strains can produce pellicle material by 24 h and it remains intact after 48 h in all strains except those lacking Pea EPS (Chapter 3). Thus, we were interested if the initial pellicle formation was due to purlin production. Pellicles formed in static one-day old cultures by wild-type, Δ*psgA,* Δ*bcs,* and Δ*bcs*Δ*psgA* mutants were robust and were easily transferred to a petri dish containing saline solution (Fig. 4B). In contrast, pellicles formed by Δ*pea* and Δ*bcs*Δ*pea* cells were thin and broke apart upon transfer of the pellicle material, although we were still able to do so. This was not the case for the Δ*pea*Δ*psgA* and

Δ*bcs*Δ*pea*Δ*psgA* pellicles as they were even thinner than their parental mutant strain pellicles and disintegrated upon any transfer attempt (Fig. 4B).

Pellicles reflect the strength of cell-to-cell interactions, which is necessary for stabilization of biofilm biomass. Therefore, we used 24 h biofilm microtiter plate assays to obtain a quantifiable measure of how biofilm biomass is altered in Δ*psgA* pWspR19 strains compared to their *psgA* containing controls. This method allows us to ascertain possible differences that may go unnoticed in pellicle assays. Pair-wise comparison analysis of

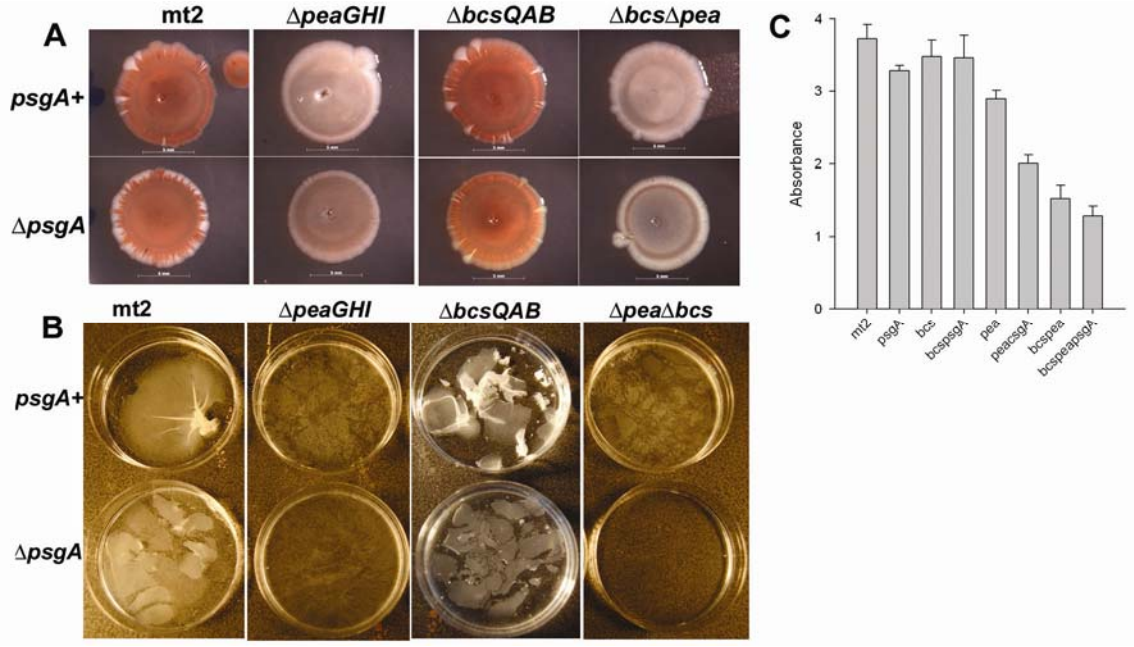

Figure 4. Biofilm formation properties of *P. putida* and purli-deficient strains expressing pWspR19. (A) Congo red binding phenotype of colonies on LB agar containing coomassie blue and congo red after 7 days. (B) Stability of pellicles formed in static cultures 24 h after inoculation in TYE broth amended with 0.3M NaCl. Pellicles were formed in a 6-well microtiter plate and then transferred using a wooden applicator stick to petri dishes filled with 0.89% saline solution. (C) 24-hour microtiter plate biofilm assays. Congo red binding of select colonies after 48 hours and development of petite colonies within Δ*bcs*Δ*pea* colony is shown in appendix C.

preliminary experiments found *psgA* removal from wild-type and Δ*bcs*Δ*pea* mutant strains significantly decreased their biofilm biomass compared to their counterparts containing the *psgA* gene (Fig. 4C). No difference was detected between Δ*bcs* and Δ*bcs*Δ*psgA* biofilm cells. The greatest reduction in biofilm biomass was noted with Δ*bcs*Δ*pea*Δ*psgA* strain compared to Δ*bcs*Δ*pea,* substantiating our pellicle assay observations. Together, these data suggest

that purlin synthesis itself is not required for the initiation or formation of young pellicles, however, an interaction between purli, Pea, and Bcs is necessary for wild-type pellicle formation to occur. Furthermore, purlin synthesis contributes to, but is not essential, for increasing biofilm biomass.

Biofilm formation on glass coverslips and microtiter plates

The original interest for this work came from the previous observation that the biofilm surface area coverage of glass coverslips by Δ*bcs*Δ*pea* mutants was comparable to the wild type and Δ*bcs* mutants, but considerably more than the Δ*pea* mutant (See Table 4 in Chapter 3). Furthermore, the pea mutant phenotype was partially restored in Δ*bcs*Δ*pea* mutants complemented with Bcs. Also, the percentage of biofilm surface area coverage was reduced, but still present after incubation of the Δ*pea* mutant biofilms with cellulase to degrade the Bcs EPS (Chapter 3) suggesting that biofilm formation was not due to Bcs and Pea polymers alone. To determine if these results were due to purli we assessed biofilm formation on glass coverslips at the air-liquid interface using mini*Tn7gfp*$_{AAV}$-tagged strains (Table 1). Multiple microscopic viewing fields collected by scanning across the width of an individual glass coverslip determined the percentage of surface area coverage per viewing field ranged from 37 to 98 percent in Δ*pea*Δ*psgA* biofilms (data not shown). Together, this led to the high error term surrounding the average percent coverslip area across all samples of Δ*pea*Δ*psgA* mutants, and to a lesser extent the Δ*bcs*Δ*pea*Δ*psgA* data (Table 2). We observed a slight, albeit non-significant, decrease in surface coverage area by Δ*bcs*Δ*pea*Δ*psgA* compared to Δ*bcs*Δ*pea* mutants. This suggests that purli production has a complex role in biofilm formation and cannot fully explain the observation of increased surface area coverage by Δ*bcs*Δ*pea* mutants. Biofilm formation is related to the ability of the bacterium to initially attach to a surface, and thus we explored if purlin and EPS are important for early phases of attachment to an abiotic surface. Although preliminary, our

Table 2. Biofilm formation by 24-h old *P. putida* mt2 and EPS-deficient mutants with or without *psgA* deletions on glass coverslips at the air-liquid interface

	Mean % surface area coverage ± SEM[a]	
Strain	*psgA*+	Δ*psgA*
mt2	94.2 ± 1.5[A]	93.9 ± 1.5[A]
Δ*bcs*	94.5 ± 3.3[A]	92.7 ± 2.6[A]
Δ*pea*	53.1 ± 4.1[C]	78.1 ± 7.3[B]
Δ*bcs*Δ*pea*	90.1 ± 3.9[A]	86.3 ± 4.1[AB]

[a] Values followed by the same letter are not significantly different (P≥0.05, LSD).

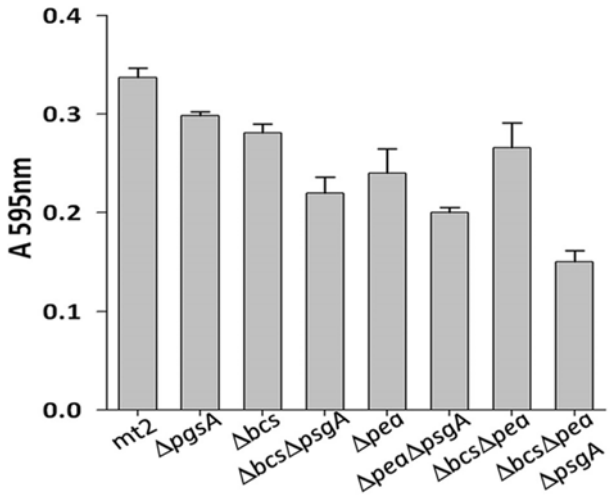

Figure 5. One hour cell attachment assay of *P. putida* mt2 and mutants. Preliminary results based on one experiment containing two replications.

findings suggest that *psgA* deletions reduces attachment to microtiter plate wells in a 1-hour assay; this experiment needs to be repeated (Fig 5). Together, this data suggests that, in addition to having a complex role in contributing to mature biofilm matrix stability, purli contributes to initial cell attachment.

Differential effect of solute and matric stress on psgA expression

Since purli have a role in biofilm formation we were interested in determining if *psgA* expression levels are altered during growth in the water-replete, osmotic, and matric stress conditions since biofilm matrix composition and the amount of EPS produced differs between treatments, thus the need for purli as a matrix component may also vary (Chang *et al.*, 2007). As can be seen in Figure 6, *psgA* expression was elevated by both water stress conditions and this expression pattern parallels that of *alg8* expression. We explored the

possibility that *psgA* expression was controlled by the AlgU sigma factor like *alg8* but were unable to identify an AlgU sigma-factor binding sites in the proposed promoter regions of *psgA* or the adjacent *psgYZB* operon (data not shown).

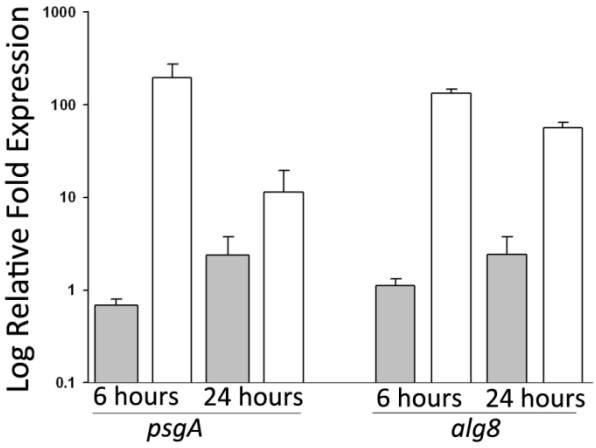

Figure 6. Effects of a 1.5 MPa reduction in water potential imposed by NaCl (gray bars) or PEG M.W. 8000 (white bars) on *psgA* and *alg8* expression in 6 and 24 h- old mt2 biofilm cells. Error bars reflect the standard deviation from two independent experiments.

Mucoid colony appearance and PalgD-gfp expression during water-limitation

Mutant strains were also spot inoculated onto water-replete and high osmolarity medium to compare colony morphology of EPS and purli mutants. During growth under matric stress conditions all strains appeared mucoidy, which is indicative of alginate production, except the Δ*bcs*Δ*pea*Δ*psgA* mutant. This phenotype concerned us since the changes of an, unknown secondary mutation increases when multiple genetic manipulations and pyramiding occur. Attempts to electroporate pN-PsgA into Δ*bcs*Δ*pea*Δ*psgA* cells were unsuccessful so a second, entirely independent Δ*bcs*Δ*pea*Δ*psgA* strain was completed by pyramiding deletions in the reverse order from the first Δ*bcs*Δ*pea*Δ*psgA* strain. This second mutant also exhibited this non-mucoid phenotype on water-limiting media. We used the P*algD-gfp* transcriptional fusion reporter to determine whether the lack of mucoidy was a consequence of no alginate gene expression (Chang *et al.*, 2007). Expression of P*algD-gfp* was low in all strains under water-replete conditions except by Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* mutants where it was elevated (Fig. 7A). As anticipated, growth in matric stress conditions greatly increased alginate expression in all

strains except the Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* mutants; this is more evident when the ratio of GFP fluorescence during matric stress conditions was compared to water-replete conditions (Fig. 7B). Expression of the pP*algD-gfp* reporter in Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* strains was also uncharacteristically high during hyper-osmotic growth conditions (data not shown). These results suggest that the lack of mucoidy exhibited by both Δ*bcs*Δ*pea*Δ*psgA* and Δ*bcs*Δ*pea* mutants under water-limiting conditions is not a consequence of the inability to express alginate biosynthesis genes but rather to synthesize or export alginate itself. Alternatively, alginate may be produced but increased alginate lyase may cause the degradation of alginate and prevent its accumulation. Lastly, non-mucoidy cells could result from changes in the fatty acid portion of the lipid membrane causing membrane instability and decoupling of AlgU from its membrane anchored MucA. To ascertain if membrane fatty acids differ significantly in EPS and purli mutant combinations compared to wild-type cells were grown in the presence of water stress and water replete conditions prior to isolating fatty acids and their analysis via gas chromatography. While there were some statiscially significant changes in the types of fatty acid proportions no consistent trend was apparent leading us to conclude that there are not suffiecent changes in fatty acids porportin leading to greater mambrane fluidity in mutant lacking combinations of EPS and purli. Thus, increased alginate gene expression was not a consequence of altered membrane fatty acid changes (see Appendix B).

Discussion

Amyloid proteins were traditionally studied for their medical relevance in diseases such as Parkinson's and Alzheimer's, although amyloid fiber formation in humans is atypical and the cause is debatable (Glenner and Wong, 1984, Conway *et al.*, 2000, Hardy, 2006). Medical research was aided by the discovery of 'functional' amyloids in *E. coli* called curli (Fowler *et al.*, 2007). Once thought to be a unique trait, amyloid proteins are ubiquitous in natural biofilms and contribute to biofilm stability and pellicle formation in *B. subtilis* (Larsen *et al.*, 2007, Romero *et al.*, 2010). In some bacteria, amyloid proteins are associated with

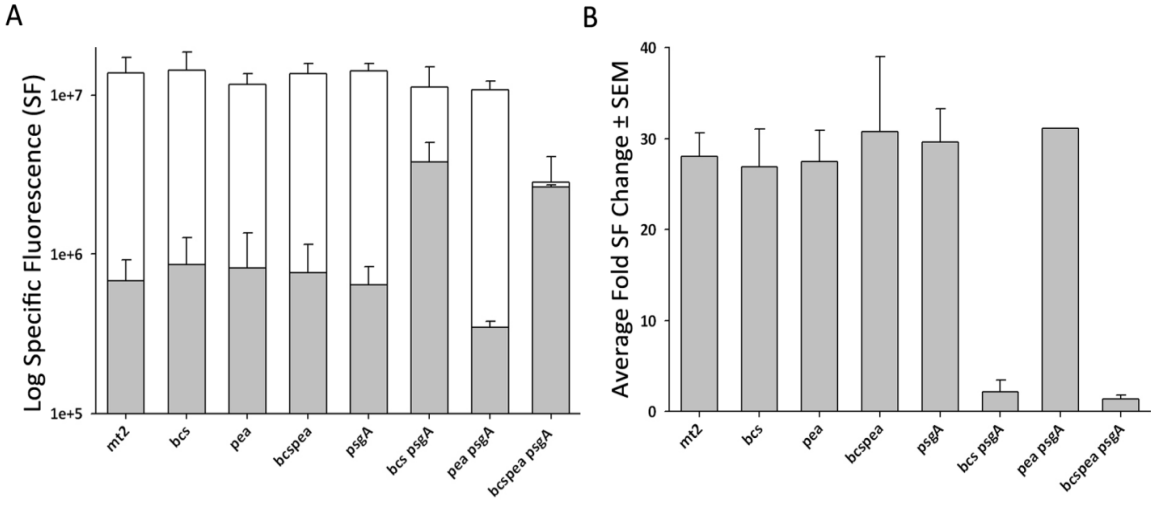

Figure 7. Effect of *psg*A and EPS deletions on the specific fluorescence measured in strains harboring the *algD* promoter-*gfp* transcriptional fusion (pP*algD-gfp*) under water-replete and -limiting conditions. (A) Biofilms were cultivated for 24 h on media without (gray) and with PEG M.W. 8000 (white) amendments to lower the water potential by 2.5 MPa. Specific fluorescence is defined as the fluorescence intensity divided by culture OD_{600} nm. (B) Average fold induction of pP*algD-gfp* expression during water-limiting relative to water-replete conditions. Where present, bars indicate the SEM of at least three independent experiments.

cellulose suggesting that the biofilm matrix is a highly ordered structure with specific associations between cells, exopolysaccharides, and proteins. In others, EPS and adhesins have overlapping roles which they exert in a compensatory manner suggesting that either they are co-regulated or the presence of one is physically masked by the other.

Based on previous assessment that a mutant of Bcs and Pea restored stable biofilm adherence to glass coverslips we wondered if other adhesins were responsible. Candidates included LapA, a protein shown to be important for the transition to irreversible cell attachment in phosphate replete conditions, FabC, a newly discovered amyloid-like protein, and PsgA, an *E. coli* CsgA homolog (Monds *et al.*, 2007, Dueholm *et al.*, 2010). We choose to explore purlin, encoded by the *psgA* gene, since *S. enterica* and *S. coelicolor* cells suggest their amyloid-like proteins interact with cellulose and *P. putida* produces two cellulase-sensitive polymers, Pea and Bcs (Chapter 3)(White *et al.*, 2006, De Jong *et al.*, 2009). Isolation and formic acid treatment of proteins sheared from the *P. putida* outer membrane found a protein that reacted with WO1 antibodies and increased the fluorescence intensity

of Thioflavin T dye. Our ability to detect WO1 binding required over expression of purli and may explain why the curli-producing *E. coli* MC4100 strain did not react. Furthermore, *psgA* expression was unable to complement an *E. coli csgA*- mutant since it was unable to bind WO1 and colonies of *E. coli csgA*- pN-PsgA cells did not bind congo red (data not shown). Assembly of amyloid-like fibers requires a nucleation protein that shares specific residue repeats with the major amyloid subunit protein. Although *E. coli* and *P. putida* have the nucleation proteins CsgB and PsgB, respectfully, these two may not be interchangeable with one another and provide one reason why PsgA polymerization in *E. coli* was not possible. PsgA also binds the fluorogenic dye Thioflavin T since cells that lack the ability to produce PsgA could not. This suggests that purli fibers are produced and, if other amyloidogenic proteins are present, such as FabC, they either did not contribute significantly to binding under the tested conditions, were not produced, or were not in the correct conformation required for Thioflavin T binding after formic acid treatment.

The role of PsgA in biofilm formation is complex and likely involves interactions with multiple EPS polymers, additional proteins or adhesins, or DNA present in the *P. putida* biofilm matrix. Similar to *E. coli* biofilm cells (Gualdi *et al.*, 2008), independent removal of PsgA and Bcs EPS genes results in a modest decline in cell attachment compared to wild-type cells, however, attachment was further decreased, but not eliminated, in Δ*bcs*Δ*psgA* cells. While these results are intriguing, it should be kept in mind they are preliminary and the experiment needs to be repeated. That stated, PsgA appears to have a further integrated role with the Pea EPS polymer in initial pellicle formation and adherence to microtiter wells in WspR19 expressing cells. A model in which both Pea and purli serve dual roles as surface and cell-to-cell adhesions, yet also interact with one another, is possible. In this model, removal of either purli or Pea would decrease the cell-cell and cell-surface adherence of cells needed to maintain pellicle stability and biofilm biomass, and hence, removal of both polymers would result in a greater decrease than each one independently. However, this model does not take into account our coverslip adherence assay results in which Δ*pea*Δ*psgA* biofilm covered more surface coverage area than Δ*pea* biofilms. To

explain these results we must consider two points: first, previous experiments showed that the amount of total exopolysaccharide material was unaltered by absence of another EPS, suggesting that a compensatory EPS network exists, and two, other adhesin proteins are known. The most likely candidates are Peb exopolysaccharide and the LapA protein, although FabC, the other amyloidogenic protein in *P. putida* cannot be fully ruled out as well. Both FabC and Peb may be responsible for the heterogeneous congo red binding observed over time in Δ*bcs*Δ*pea*Δ*psgA* colonies. Our lab does not currently have Peb, LapA, or FabC, mutants to test these possibilities. Together, our data highlights the complexity of interactive matrix components needed for formation and stability of the *P. putida* biofilm matrix.

Formation and stability of the biofilm matrix is particularly important in low-water-content habitats since dehydration stimulates alginate production, which facilitate cell hydration. During low-water content conditions the amount of alginate increases proportionally to the level of dehydration the cell senses, to a point that it is sufficiently hydrated, or to a point that the cell becomes limited in the resources necessary to produce alginate. Alginate is capable of holding several times its weight in water implying that an increase in alginate, along with its water sorption properties, places weight constraints on the biofilm cells and may require more structural support. This may explain why we noted similar increases in *alg8* and *psgA* gene expression under growth in low-water content conditions. However, further experiments will need to be performed to determine if the EPS matrix contains more purli under water-limiting conditions since expression does not always result in greater purli synthesis. Especially when the availability of the CsgA curli protein in *E.coli* can be limited by interactions with the CsgG lipoprotein; *P. putida* contains the CsgG homolog (Loferer *et al.*, 1997).

Since alginate is used as a primary mechanism to hydrate the biofilm matrix it was surprising to find that two independently constructed Δ*bcs*Δ*pea*Δ*psgA* mutants appeared non-mucoidty on under water-limited conditions. This was clearly a three-way interaction between Bcs, Pea, and PsgA since Δ*bcs*Δ*psgA,* Δ*pea*Δ*psgA,* or Δ*bcs*Δ*pea* cells remained

mucoidy. It was unclear why then that only Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* cells had greater alginate expression under water-replete conditions compared to all other strains, including Δ*pea*Δ*psgA.* Earlier we proposed that the non-mucoidy phenotype of Δ*bcs*Δ*pea*Δ*psgA* could be due to increased alginate lyase production or the inability to synthesize or export alginate itself. However, if either of these speculations is correct it likely results from altered cell envelope stability or increased accumulation of factors that stimulate an envelope stress response since alginate gene expression, elicited by the cell envelope stress response, is increased in these strains under water-replete conditions. Thus, the envelope stress response can trigger alginate production in Δ*bcs*Δ*psgA* cells during low-water content conditions but not in cells compromised in Pea, Bcs, and PsgA. This could result from the inability of Δ*bcs*Δ*pea*Δ*psgA* cells to produce the energy required for alginate synthesis or altered post-transcriptional regulation of either alginate production or alginate lyase activity. Likewise, the alginate biosynthetic and transporter machinery may be defective. It will be interesting to see if other components of the envelope stress response are also stimulated under water-replete or high-osmolarity conditions due to the absence of Bcs and purli polymers giving further support for altered cell envelope stress responses. Appropriate envelope stress responses are needed to retain optimal bacterial fitness and survival during water-limiting conditions and, in *Salmonella*, lack of both cellulose and tafi fibers decreased desiccation tolerance and persistence (White *et al.*, 2006). These results suggest that removal of cellulose and ALF may uncouple normal regulatory frameworks that are required for desiccation tolerance and survival. Hence, since purli seems to influence biofilm properties and alginate production, and could have a role the envelope stress response, future work will need to explore if purli contributes to desiccation tolerance and biofilm structural support.

Material and methods

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. Jensen's Medium (JM) plus 20mM Mannose was used as the growth medium for biofilm formation assays (see Chapter 3). Complementation assays were performed using the plasmid pN (Chen *et al.*, 2010) containing a full copy of the *psgA* gene. For isolation of purli proteins from the biofilm matrix, cells were cultivated on YESCA plates amended with agar (Chapman *et al.*, 2002) at 28 °C. *E. coli* TOP10 cells used as a host strain for isolating plasmids was grown in TSB at 37 °C. CTYE medium was used during P*algD-gfp* assays. When necessary the following antibiotics and concentrations were used: 50 μg mL^{-1} gentamycin (Gm), 50 μg mL^{-1} ampicillin (Ap), 15 μg mL^{-1} tetracycline (Tc), 50 μg mL^{-1} kanamycin (Km) and 35 μg mL^{-1} rifampicin (Rf).

DNA manipulation and mutant construction. Plasmid and genomic DNA was isolated from *E. coli* or *P. putida*, respectively, with the Qiagen mini-prep and Wizard Genomic DNA kit (Promega). Phusion High-fidelity Taq Polymerase (FinnEnzymes), genomic DNA, and primers PsgA1F and PsgA1R were used to PCR amplify a 4213 bp product containing the *psgA* gene and the flanking 1499 bp upstream and 1269 bp downstream regions and blunt endligated into pTok2SacB vector using T4 Ligase (Promega) to create pTok2SacB+PsgA. Inverse PCR using Phusion Taq and PsgA2F and PsgA2R dephosphorylated primers were used to amplify pTok2SacB and all regions surrounding the *psgA* gene to generate pTok2SacBΔPsgA. Amplicons were circularized by ligation, transformed into *E. coli* Top10 cells (Invitrogen), and isolated for DNA sequence analysis before electroporation into *P. putida* and selecting for Tc resistance. Tetracylcin resistant colonies were sequentially screened on TSA containing Rf and 10% sucrose for Tc^S colonies. Colony PCR using external primers PsgAUp and PsgADn verified *psgA* gene deletions and in some strains, the PCR product was sequenced. All primers used in this study are listed in Table 3.

Table 3. Primers used in this study

Name	Sequence (5'-3')
psgA1F	AAGCATCACGGTCAACGATTTGGC
psgA1R	ATCGGCTTATTGCAGTTCCAGCAG
psgA2F	ACCGCTCCCCCTTGAACC
psgA2R	GGTGGTGCCTCCAGGTCTATC
psgAUp	TTCGATGAAACGGCTGTACTCCCTG
psgADn	ATCGGTAGCCATGTTGATCACTCACC
psgA-FPr	TGATCGAGCAACAAGGGTTAGGCA
psgA-RPr	TCTGCTGGATATCGCTGGTGCTTT
psgAInF	CGATATCCAGGTCTATCAGGAAGG
psgAInR	GTAATGGTGGTGGTCGAGTTGTTG

Operon and promoter prediction tools. Potential ribosomal binding sites were identified by comparison of the *psgA* and *csgB* intergenic region with the *E. coli* Shine-Delgarno consensus sequence. Sigma-70 promoter prediction was performed using the BPROM software while potential rho-independent terminators were identified with FindTerm (www.softberry.com).

To verify BRPOM promoter predictions, reverse transcription PCR (RT-PCR) was performed using primers psgA-FPr and psgA-RPr which were designed to flank the predicted *psgA* promoter site and *psgA* translational start site. Internal primers psgAInF and psgAInR were used to verify the presence of a *psgA* transcript. RNA was isolated from cells exposed to water replete, hyperosmotic, or water-limited conditions for 6 h using the Qiagen RNA extraction kit followed by Turbo DNase digestion (Ambion). Quanta q-Script 1-step reaction mix (VWR) without Sybr Green and with or without reverse transcriptase was used for RT-PCR under the following conditions: 55 °C 10 m, 95 °C 5 m, (95 °C 30 s and 60 °C 20 s) x 40 cycles, 72 °C 1 m. Products were visualized by agarose (0.8%) gel electrophoresis.

Congo red and pellicle assays. Five µl aliquots of overnight TYE-grown cultures were spotted onto ½ TYE-H plates containing 0.3 M NaCl, 80 µg/mL congo red, and 15 µg/mL coomassie blue. Red or pink colonies on congo red plates indicated the binding of congo red to extracellular matrix material. For pellicle assays 10 µl aliquots of 100-fold dilutions of overnight TYE-grown cultures were inoculated in 20 mL of TYE media containing 0.3 M NaCl and incubated statically for 24 hours. For photography, pellicles were gently lifted off the

culture air-liquid interface using a wooden applicator stick and placed into a small petri dish containing 10 mL 0.89 % saline solution.

qRT-PCR of *psgA* expression. Stationary phase cells inoculated from individual colonies grown on King's B agar were subcultured into fresh ½ 21C-GS broth and grown to mid-exponential phase (OD 600_{nm}= 0.4). Cells were centrifuged and suspended into pre-warmed ½ 21C-GS medium and incubated an additional 15 minutes before inoculating water replete or water limiting (-1.5 MPa Ψ) ½ 21C-GS solid media. At appropriate times, bacterial RNA protect (Qiagen) was added to plates containing biofilm cells and total RNA was isolated using the RNAEasy kit (Qiagen), including on-column DNase digestion and Turbo DNase digestion (Ambion). RNA was quantified using the Nanodrop 1000 (Thermo Scientific). Diluted RNA, 5 pmol primers (Table 3), and the Quanta qScript 1-step SYBR Green kit (VWR), was used along with the BioRad iCycler Q-PCR thermocycler to produce cDNA and for qRT-PCR . The $\Delta\Delta$C(t) method was used to calculate the relative fold change in expression of the targeted genes in water stress conditions compared to water replete and normalized to *rimM* expression.

Thioflavin T binding assays. Fourty eight to seventy two h-old biofilms were cultivated on YESCA plates prior to suspension of cells into 100 mL of a pH 7.6 10 mM Tris-Base buffer (referred to hereafter as Tris buffer). Extracellular proteins were sheared off the cells by blending in a Waring blender three times at one-minute intervals. Samples were centrifuged twice to remove cells and supernatant proteins were precipitated with 300 g/L ammonium sulfate. Following centrifugation, precipitated proteins were suspended in Tris buffer prior to removing a 250 µL aliquot for drying using a Speed-vac concentrator. One hundred µL of an 86% formic acid solution was added to dried proteins before subsequent removal of formic acid by Speed-vac and reconstitution into 100 µL Tris buffer. Proteins were quantified using the Bradford Assay (BioRad). Tris buffer and a volume corresponding to 0.1 mg mL^{-1} formic acid resistant proteins or BSA was mixed with a concentrated Thioflavin T (ThT) solution until the final ThT concentration was 20 µM. The emission spectrum of the samples was measured on a Fluoromax-2 spectrofluorometer

using an excitation wavelength of450 nm and a 5 nm bandpass with a 0.1 second integration time. Four reads were averaged per scan and background associated fluorescence in samples containing proteins, but no ThT, were subtracted.

Biofilm assays. Pellicle and microtiter plate assays were performed as previously described in Chapter 3 using ½ TYE-H NaCl medium. For biofilm formation on glass coverslips late stationary phase mini-Tn7-gfp chromosomally tagged *P. putida* strains were inoculated into 10 mL of JM medium containing a partially submerged sterile glass coverslip and incubated upright at 28 °C with gentle agitation (50 rpm). Coverslips were removed and washed by gently dipping in sterile water three times. Epifluoresence microscopy 12-bit images were obtained using a Nikon Eclipse 80i system equipped with a Nikon EZ coolsnap camera using the 10X objective lens and a GFP-HYQ (EX: 450-490nm, DM: 495nm, BA: 500-550nm) filter cube. Images were processed to determine the proportion of surface area covered by biofilms at the air-liquid interface using MetaVue v7.1 software (Molecular Devices) by background subtracting non-colonized areas and excluding pixels with luminescence values below that of GFP-expressing cells. Remaining pixels were counted using the 'region measurements' tool and data was transferred into an Excel spreadsheet to calculate the percentage of surface area coverage by dividing non-background associated pixels by total number of pixels. A one-way ANOVA was performed using JMP 8.0 by pooling multiple experiments with at least three replicates per experiment.

P*algD-gfp* bioreporter studies. The pP*algD-gfp* reporter plasmid was electroporated into *P. putida* Δ*psgA* strains. Cells were grown for 18 h on CTYE plates prior to suspension and dilution in CTYE broth to an OD_{600} of 0.1 and further diluted 100 to 1000-fold prior to inoculating 5 µl aliquots onto 0.45 µm pore-sized nitroceluloase membranes overlaid onto CTYE (water replete) or -2.5 MPa Ψ PEG8000-amended plates. After 24 h, membranes were removed from the plates and suspended into two mL of a 0.89% saline solution. The OD_{600} was recorded and GFP fluorescence intensity was quantified with a Fluoromax-2 spectrofluorometer. Excitation and emission wavelengths were 488 and 510 nm, respectively with a 5 nm bandpass and 0.1 second integration time.

FAME analysis. Cells were cultivated on nylon membranes overlaid onto CTYE medium with and without lowering the water potential by NaCl or PEG8000 amendments for 24 h at 28 °C. Fatty acids were isolated according to the previously published methods (Bligh and Dyer, 1959) and analyzed by gas chromatography using the manufacturer's recommended protocol (MIDI system, Newark, Del.) and compared to known a mixture of 16:1 ω7*t* and 16:1 ω7*c* standards (Sigma-Aldrich).

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Chapter 5. Microarray comparison of *Pseudomonas putida* gene expression changes during a sudden reduction in water availability by a solute or a matric shock

Summary

Bacteria in terrestrial habitats frequently undergo fluctuations in water abundance. Reduced water availability can occur by two means: the sorption of water to the surrounding abiotic surfaces in low-water content environments (matric stress) or by high salt concentrations (solute stress). Effects and responses to hyperosmotic conditions are well documented, however, matric stress, is largely overlooked. Transcriptome analysis of *P. putida* KT2440 was performed to provide insights and compare the effects and responses to reductions in solute or matric-mediated water potentials. While both solute and matric shock resulted in many similar altered gene expression patterns, the extent of induction was more dynamic during matric conditions. A total of 275 genes had significantly higher gene expression values following a water stress shock compared to water-replete conditions. Seventeen of these genes had expression values that were within 25% of one another while sixteen were expressed more in solute- than matric-stress conditions. Induced genes included those responsible for compatible solute accumulation, heat-shock proteins, and extracytoplasmic function (ECF) sigma factors. Additionally, gene expression of Arginine Deiminase (ADI) pathway and TCA-cycle were altered suggesting central metabolic network changes. The *phoP/Q* and *oprH* genes were induced solely by solute shock. Far fewer genes were repressed than induced including mobile elements, succinate dehydrogenase, motility, and ATP synthase subunit genes. Certain genes that had significant gene expression changes based on microarray results were further targeted for qRT-PCR analysis to determine if early gene expression during shock conditions were predictive of events occurring in biofilm cells grown in osmotic and matric conditions.

Together, our data suggests that many water stress responses overlap but matric stress is far more stressful than the thermodynamically equivalent solute stress.

Introduction

Water is necessary for sustaining life and its availability to soil microorganisms fluctuates between saturated and unsaturated conditions. The amount of freely available water relative to pure water, defined as water potential (Ψ), is influenced by two primary components: the concentration of solutes and the amount of water physically bound to surfaces by electrostatic or capillary forces during low-water content conditions, known as the matric component (Potts, 1994). Sudden reductions in water potential by increased solute or matric forces cause water loss from the cytosol and a reduction in cell turgor pressure. Loss of turgor pressure and cytosolic water can damage, or cause the aggregation of proteins, alter the structure and functions associated with the cell membrane, and destabilize the cell envelope (Billi and Potts, 2002, Or *et al.*, 2007). Increasing cytosolic water content can abate these effects. Responses to both the initial exposure to, and growth in the presence of, low water potential conditions require continuous energy input. Studies comparing differential responses to solute and matric stress have mainly centered around long-term acclimation responses rather than initial responses to a sudden change in water availability (van de Mortel and Halverson, 2004). This might be due to the difficulty of isolating transient initial shock responses and further reflects the assumption that differences between solute and matric stresses are similar during initial acclimation phases (Adebayo and Harris, 1971, Howie *et al.*, 1987, Record *et al.*, 1998). This may stem from the fact that during solute conditions, the cell is bathed in water of diminished activity, whereas cells growing in matric conditions are considered dehydrated since they are surrounded by a very thin (10-400 molecules thick) water layer (Harris, 1981). Dehydration imposes additional stressors on the cell such as nutrient limitations, since water transports dissolved nutrients to the cell, and solute stress, as external solutes are concentrated as environmental water content decreases (Adebayo and Harris, 1971, Potts, 2000, Or *et al.*,

2007). Although matric and solute stress may be thermodynamically equivalent their effects on cells are likely not.

In Pseudomonads and other bacterial species, the responses to solute stress are well documented. Cell responses to solute stress is a step-wise process triggered by sensing a reduction in turgor pressure, followed by potassium ion uptake and accumulation of compatible solutes (Epstein, 1986, Csonka, 1989, Garay-Arroyo *et al.*, 2000, Sleator and Hill, 2002). Recently, *P. aeruginosa* was shown to induce genes encoding hydrophilin proteins in response to solute stress, but their mechanism of action is unclear (Aspedon et al., 2006). Wide ranges of compatible solutes exist and the types utilized are dependent on the bacterial strain, nutrient source, and which other compatible solutes have already accumulated (Kets *et al.*, 1996, Freeman *et al.*, 2010, Kurz *et al.*, 2010). For example, microarray analysis of *P. aeruginosa* cells following a sudden solute up-shock found that many compatible solute synthesis pathways or transporter genes were induced, with the exception of choline (Aspedon et al., 2006). In contrast, *P. syringae* increases choline transporter gene expression during water stress conditions (Peterson, 2009, Kurz et al., 2010). To date, *P. putida* has been demonstrated to produce trehalose, mannitol, glycine betaine, NAGGN, and glutamate in response to growth in solute conditions (Kets *et al.*, 1996, Kurz *et al.*, 2010). It is unknown which, if any, compatible solutes or hydrophilins are produced by *P. putida* in response to a solute or a matric shock.

Reduction in water potential elicits numerous other cellular responses linked to the damage caused directly or indirectly by water deprivation. Microarray analysis of solute stressed *P. aeruginosa* cells identified increased expression of genes associated with envelope proteins, such as cytochromes, periplasmic proteases, two-component systems, AlgU and other ECF sigma factors (Aspedon et al., 2006). Previous *P. putida* transcriptional reporter assays found gene expression of several proteases, chaperones, genes encoding envelope-associated proteins are induced during growth in matric conditions (van de Mortel and Halverson, 2004). Additionally, protein and DNA damage response pathways are elicited, and in some cases, shown to be a direct cause of water stress (Mattimore and Battista, 1996, Dmitrieva and Burg, 2007). While some general stress responses may overlap

during growth in matric and solute stress no direct, in-depth comparison of the initial water stress responses have been done.

The objective of this study was to gain an understanding of the gene expression responses that occur during the early stages of adaptation to thermodynamically equivalent solute and matric stress and to determine which aspects of the response are specific or shared by the two stresses. Our approach was to use high density oligonucleotide microarrays to investigate the transcriptional responses of *P. putida* KT2440, a TOL-plasmid free derivative of mt2, to both solute and matric shock stress conditions. We capitalized on this information to investigate whether these short-term adaptations are continued during growth of *P. putida* biofilms during similar stress conditions and found gene expression is similar under shock conditions and during growth in the same stress for several, but not all, genes. In general, matric stress causes a greater change in gene expression levels compared to solute stress. This, and the fact that matric stress uniquely alters the expression of more genes than solute stress, implies that matric stressed cells perceive a greater stress than the thermodynamically equivalent solute stressed cells.

Results and Discussion

Overview of transcriptional response to water stress

Microarray gene probes were based on the 5,283 Glimmer 2 predicated functional genes in *P. putida* KT2440 and did not include RNA coding genes or any of the 67 genes suggested by www.tigr.org to produce "corrupt protein products due to frame shift or point mutations". In total, 439 out of the 5,283 (8.3 %) genes were differentially expressed upon matric or solute shock treatment using a mixed-model ANOVA analysis with the criteria of $p \leq 0.05$, a False Discovery Rate (q) ≤ 0.10, and ≥ 1.5 fold expression value change relative to water-replete conditions (Appendix E,F). Of these, sixty-two percent are solely altered by matric shock, 2 % by solute shock, and the remaining 56 % were altered by both shock treatments. More genes are induced than are repressed by decreased water availability. Thirty-three percent of the up-regulated genes are solely induced by matric conditions, 3 %

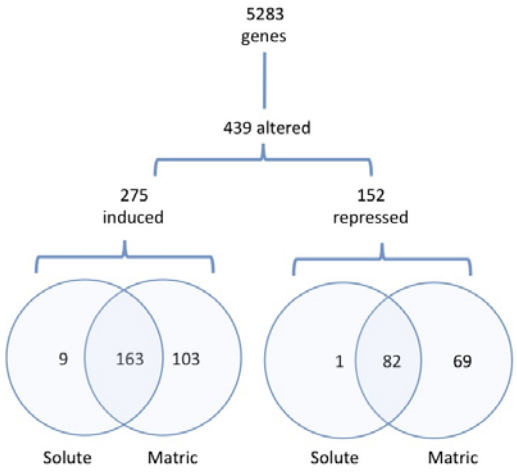

Figure 1. Number of *P. putida* genes whose expression was altered 1.5-fold or more, relative to water-replete conditions, after a 15 minute exposure to a 1.0 MPa water potential reduction by either matric or solute shock treatment.

by solute shock, and 59 % are co-induced. Out of the 152 down-regulated genes, 45 % are unique to matric while slightly less than 55 % are decreased by both stresses (Fig. 1, Appendix F). Only one gene is repressed solely by solute shock. The majority of genes whose expression was uniquely altered by matric stress do not have predicted functions.

The location of proteins encoded by genes that alter their expression in response to water stress gives clues about which cellular compartments may require modifications. PSORTB v3 analysis predicts 1,515 proteins are located in the cytoplasmic membrane, periplasm, and outer membrane compartments, referred to as the cell envelope, while 2,402 proteins are cytoplasmic (Yu *et al.*, 2010). Of the 439 differentially expressed genes 141 encode proteins localized to an unknown location, 193 to the cytoplasm, 77 to the cytoplasmic membrane, 14 to the periplasm, and 14 to or associated with the outer membrane (Table 1). Only one gene encoding a protein of unknown location was uniquely repressed by solute shock while 77 were uniquely regulated by matric shock. A similar proportion of total genes encoding proteins localized to the cytoplasm (193/2,402) or the cell envelope (105/1,515), calculated from the sum of all genes encoding cytoplasmic membrane, periplasm, or outer membrane proteins, alter their gene expression patterns in

Table 1. Percentage and total number of differently expressed genes sorted according to predicted protein localization after shock treatment

Location [a]	Transcriptional change	% Both [b]	% Matric [b]	% Solute [b]	Total [c]
Cytoplasm	Repressed	52	48	0	60
	Induced	65	35	0	133
Cytoplasmic membrane	Repressed	46	54	0	37
	Induced	50	50	0	40
Periplasm	Repressed	33	67	0	3
	Induced	64	36	0	11
Outer membrane	Repressed	67	33	0	3
	Induced	73	27	0	11
Unknown	Repressed	57	41	2	53
	Induced	64	36	0	88

[a] Predicted protein localization based on PSORTB v3.0

[b] Percentage of significantly changed transcript levels from water replete conditions; Both refers to altered gene expression during matric and solute stress

[c] Total number of induced or repressed transcripts across all treatments

response to both water stresses. Since the expression of a majority of genes encoding proteins localized to the cytoplasmic membrane and periplasm compartments are affected only by matric stress implies that matric shock elicits the need for more unique cell envelope modifications than solute shocked cells.

We further classified differentially expressed genes according to their predicted roles by the J Craig Venter Institute (JCVI.org) based role categories and those we defined to highlight specific functions, as described the legend to Fig. 2. Osmoadaption and protein fate gene classes only appear in Fig. 2A since these genes were only induced. Genes belonging the ATP generation, motility, or mobile elements classes were only repressed and therefore unique classes in Fig. 2B. All other classification groups contain both up and down-regulated genes and therefore appear in both Fig. 2A and 2B. Interestingly, there were always more genes that were induced than repressed between these shared classes (compare Fig. 2A to Fig. 2B). Excluding genes encoding hypothetical proteins, the majority of induced genes belong to the groups involved in metabolism, regulation, and protein fate (Fig. 2A). The fourth largest class of induced genes encodes cell envelope proteins and is under represented since many genes were placed in other categories based on other

predicted functions (i.e. transport and binding). The classes containing the greatest number of repressed genes were the mobile elements (Fig. 2B). Most striking is the number of genes uniquely expressed by matric stress and belonging to the metabolism group. This, as well as similar responses seen within other gene classification groups, implies that this could be a consequence of the number of uniquely induced regulatory genes during matric shock conditions.

Water stress alters expression of regulatory genes and their targets

Expression of several regulatory systems are affected by matric and solute shock, such as, ECF sigma factors, H-NS family MvaT proteins, regulatory RNAs, and two-component systems. While many of these regulatory systems are induced by solute and matric stress, matric stress tends to elevate expression more than solute stress and, in some cases, based on qRT-PCR analysis, induction of regulatory genes continues during growth under matric, but not solute stress conditions.

Expression of the ECF sigma factor gene, *algU,* was highly induced in both water stress conditions; although it was induced nearly 2-fold more in matric shock conditions (Table 2). Additionally, the genes encoding the P*algD* enhancer-binding proteins, AlgB and AlgR, increase specifically during matric conditions which is consistent with previous evidence that alginate biosynthesis genes and alginate are uniquely produced during matric stress (Chang et al., 2007). To establish whether our microarray results translate into predictive behaviors during biofilm growth under water stress conditions we followed their expression during biofilm development by quantitative real-time PCR [qRT-PCR]. *AlgU* gene expression was continuously elevated in matric stress relative to solute or water-replete conditions and these expression patterns correlate with the co-transcribed *mucA* anti-sigma factor and *alg8,* the second gene in the alginate biosynthetic pathway (Table 2, Fig. 3A-C). By 72 h, *alg8* expression was reduced compared to 6 h but was still significantly above solute stress conditions (Fig. 3C). Furthermore, since *algU* expression remains high during matric conditions and AlgU is released from its membrane bound anti-sigma

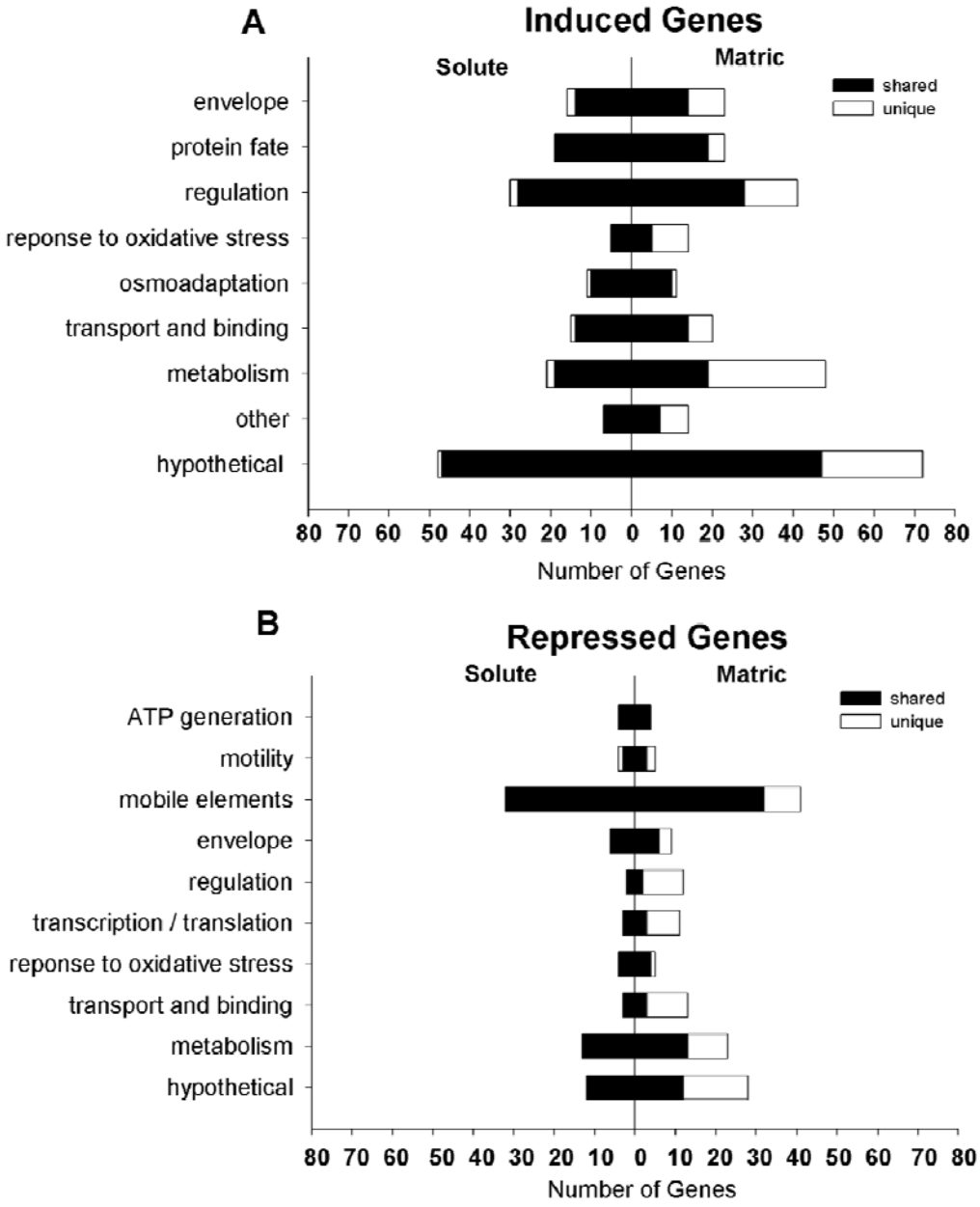

Figure 2. Classification of A) induced and B) repressed genes in response to matric or solute shock conditions. Gene classifications are based on role categories defined by the J. Craig Venter Institute (JCVI.org) and manual placement of certain genes into self-defined classes to highlight their specific functions. Classes include the following: envelope (periplasm, outer and inner membrane components), protein fate (heat shock proteins), regulation (DNA, RNA, and protein interactions), osmoadaption (synthesis or transport of compatible solute generation, mechaniosensitive channels, and putative hydrophilins), metabolism (anabolic and catabolic enzymes), motility (translocation motor and flagellar structure), mobile elements (transposases, maturases, and recombinases).

Table 2. Expression of regulatory genes

		Fold expression[1]	
Gene[2]	Predicted Function	Solute	Matric
PP0133 *algB*	Alginate biosynthesis transcriptional regulator	1.4	3.1
PP0181	DNA-binding inhibitor id-2-related protein	1.0	3.4
PP0185 *algR*	Global regulator protein	1.9	3.1
PP0194 *algP*	Transcriptional regulator	1.8	2.8
PP0216	GGDEF-containing sensory box protein	1.6	3.1
PP0246 *ompR*	Transcriptional regulator	1.5	2.7
PP0247 *envZ*	Two-component system sensor protein	1.3	1.8
PP1185 *oprH*	Outer membrane protein, OprH	38.6	1.7
PP1186 *phoP*	Two-component system response regulator	17.1	1.0
PP1187 *phoQ*	Two-component system sensor protein	2.3	-1.1
PP1427 *algU*	ECF sigma factor, σ^{22}	8.5	14.3
PP1428 *mucA*	Membrane bound AlgU anti-sigma factor	7.9	12.9
PP1429 *algN*	Periplasmic negative regulator	4.6	7.1
PP1430 *mucD*	Periplasmic protease	4.7	7.1
PP1757 *bolA*	Transcriptional activator of shape determination	3.7	6.1
PP2088 *sigX*	ECF sigma factor	5.2	6.7
PP2089 *oprH*	Outer membrane protein, OprH	1.2	1.8
PP2144	TetR-family transcriptional regulator	1.1	2.4
PP2475	TetR-family transcriptional regulator	1.5	4.3
PP2499	CopG-family transcriptional regulator	2.3	2.8
PP2947 *turE*	Transcriptional regulator, MvaT-like	2.7	4.4
PP3033	Transcriptional repressor cI/cII family protein	2.9	5.2
PP3757	Response regulator	3.4	5.7
PP3758	Response regulator	2.8	3.2
PP3759	Methyltransferase	2.6	2.9
PP3760	Chemotaxis methyltransferase	3.1	3.2
PP3761	Hybrid two-component system	2.5	3.4
PP3762	Response regulator	1.8	6.0
PP3765 *turB*	Transcriptional regulator, MvaT-like	2.2	5.0
PP3832 *csrA*	Carbon storage regulator homolog	4.3	7.6
PP4099 *gacA*	GacA response regulator	1.2	1.9
PP4470 *amrZ*	Alginate and motility regulator, P*algD* regulator	2.7	3.4
PP4894 *hfq*	RNA-binding protein Hfq	1.9	3.7
PP5047 *ntrB*	Nitrogen regulatory protein histidine kinase	-1.7	-1.8
PP5048 *ntrC*	Nitrogen metabolism transcriptional regulator	-1.6	-1.3
PP5108 *rpoH*	ECF heat shock sigma factor RpoH, σ^{32}	1.9	6.1
PP5234	Regulator of glutamine synthetase activity	-2.3	-1.9

[1]Solute or matric induced change in water potential relative to water-replete conditions.

[2]Gene numerical order and names according Glimmer2-based tigr.org genome annotation or *E. coli* homologs

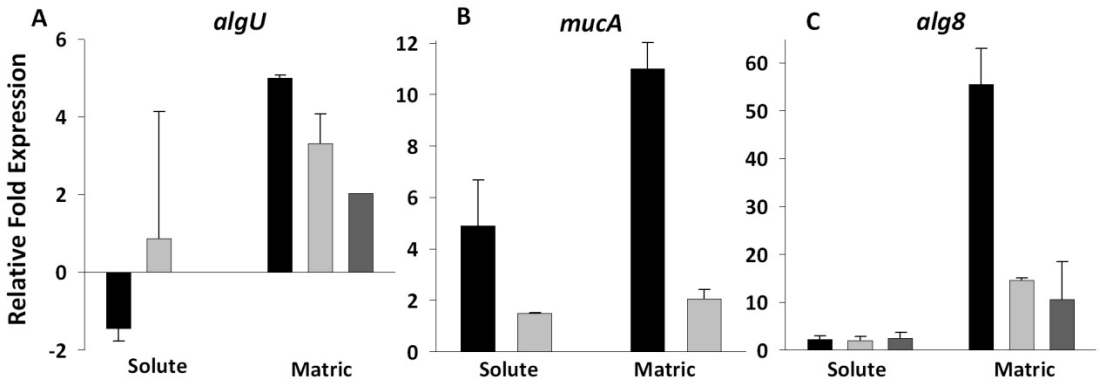

Figure 3. QRT-PCR of (A) *algU*, (B) *mucA*, and (C) *alg8* relative gene expression fold change during water stress compared to water-replete conditions in 6 (black), 24 (light gray), or 48 (dark gray) h-old *P. putida* mt2 biofilms. Water potential was lowered by 1.5 MPa using NaCl or PEG8000 amendments. Error bars indicate SEM based on three to four experiments.

factor and regulates its own expression our findings suggest that matric stress causes a persistent, rather than transient, envelope stress response.

Other polysaccharides traditionally regulated by σ^{70} are the osmoregulated periplasmic glucans encoded by the *opgGH* and *nvdB* genes in *P. putida* (Rowley *et al.*, 2006). To date, we known these glucans are produced during hypo-, but not hyper-, osmotic conditions (Talaga *et al.*, 1994, Bohin, 2000). However, we found the *opgGH* genes, but not the cyclic periplasmic glucan synthase gene, *nvdB,* are induced 3.9-fold and 2.0-fold during matric and solute shock, respectively. These results were intriguing given our unpublished data that suggests glucans may be secreted into the biofilm matrix and comprises the largest extracellular carbohydrate component under matric conditions. Thus, we wanted to validate these observations to determine if expression of both the *opgG* and *nvdB* genes are regulated differently under solute and matric stress. We found *opgG* gene expression was elevated in matric stress conditions whereas it was either repressed or equal to water-replete conditions during solute stress (Fig. 4A). These results are partially consistent with the microarray studies by Wood and Ohman (2009) that showed induction of *mdoG* after 15 minutes exposure to a cycloserine induced cell wall stress, but not a 60 minute exposure, was increased. *NvdB* gene expression levels were similar in all

conditions tested (Fig. 4B). Also, mutation of the *P. syringae opgG* gene lead to increased *algD* gene expression and is one factor required for production of the hygroscopic EPS, alginate (Chang *et al.*, 2007, Penaloza-Vazquez *et al.*, 2010). Together, the significance of these results suggests that linear glucans production is stimulated under the same conditions as needed for alginate production, suggesting it is part of the envelope stress response. Furthermore, this suggests glucans may accumulate in the periplasm or biofilm matrix and facilitate periplasmic hydration and homeostasis.

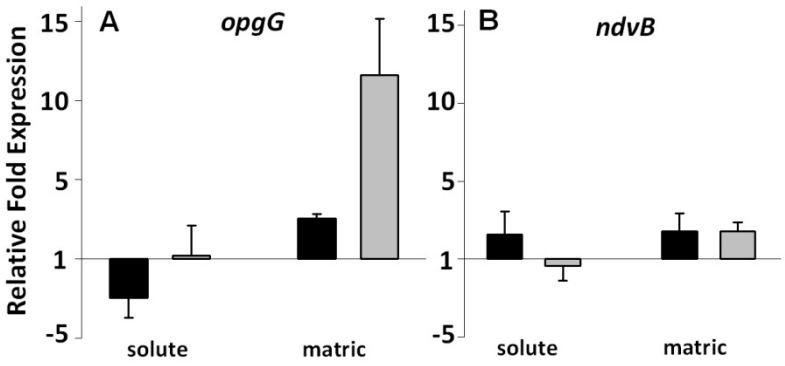

Figure 4. Q-RTPCR of the linear osmoregulated periplasmic glucan (*opgG*) and the cyclic periplasmic glucan (*nvdB*) from 6 hr (black) and 24 h-old (gray) biofilm cells growing in the presence of a -1.5 MPa Ψ solute or matric stress. Error bars represent SEM based on three to four experiments.

Gene expression of *rpoH* and the ECF sigma factor gene, *sigX,* also increased. Assuming gene expression is correlated to protein production, the concentration of alternative sigma factors increase in response to shock treatments, which could cause global changes in gene expression. In fact, gene expression of numerous genes that are controlled by RpoN, such as *glnA* (discussed below)*, ntrB* and *ntrC* regulatory proteins (Table 2), flagellar structural gene *flgB*, *dctA*, and a PP1071 periplasmic amino acid ABC transporter were decreased (data not shown)(Cases *et al.*, 2003). We also detected down-regulation of several additional genes predicated to contain RpoN-binding sites within their promoter regions, including those that are associated with pili production, nitrogen metabolism, and a methyl-accepting chemotaxis protein (data not shown) (Cases et al., 2003).

Both water stresses elevate *sigX* gene expression but *oprF* gene expression was only increased during matric shock. This was interesting given that *sigX* and *oprF* are co-transcribed in *P. aeruginosa* (Brinkman *et al.*, 1999). SigX is postulated to regulate other genes besides *oprF* since complementation of OprF in a *P. aeruginosa sigX* mutant background only partially restored growth rates in rich medium (Brinkman et al., 1999).

Expression of genes encoding other outer membrane proteins increased with water stress shock. This may be, in part, due to increased *ompR-envZ* and *hfq* gene expression that are responsible for encoding the OmpR-EnvZ two-component system and Hfq RNA-binding regulatory proteins. Both the OmpR-EnvZ system and and Hfq regulate the ratio of OmpF/OmpC outer membrane porins, mobile element genes, catalase activity, and the downstream network regulating alginate secretion through its stabilization of RpoS and other small regulatory RNAs (Sonnleitner *et al.*, 2003, Sonnleitner *et al.*, 2006, Udekwu and Wagner, 2007). Unique to solute shock is the high induction of the *phoQ-phoP-oprH* operon encoding the two-component system PhoQ-PhoP and outer membrane protein, OprH (Table 2). *OprH* transcript abundance is elevated transiently by solute shock since qRT-PCR of biofilm cells determined *oprH* gene expression was repressed by growth in water stress conditions, albeit more repression occurred during matric, than solute, stress (Fig. 5). This operon is activated upon Mg^{2+} starvation and OprH accumulation provides resistance to polycationic antimicrobials, implying that a sudden increase in cations, such as the sodium ions used for solute shock conditions, may have caused momentary displacement of Mg^{2+} and the induction of *phoP-phoQ-oprH* operon rather than reduced solute-mediated water potential per se (Young *et al.*, 1992, Macfarlane *et al.*, 1999, Cho *et al.*, 2006, McPhee *et al.*, 2006). However, further confirmation of this speculation is warranted.

The gene encoding GacA, the cognate response regulator of the GacS-GacA two-component regulatory system, increased its expression solely during matric shock (Table 2). GacS-GacA also lead to induction of many similar genes that we found to beinduced, such as the genes that lead to alginate biosynthesis, catalase, and production of the RNA binding protein, CsrA, an *E. coli* homolog (Blumer and Haas, 2000, Castaneda *et al.*, 2000, Kang *et al.*, 2004).

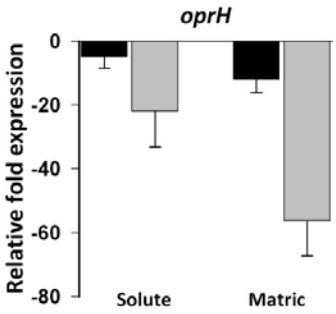

Figure 5. Q-RTPCR of the *oprH* gene from 6 h (black) and 24 h-old (gray) biofilm cells growing in the presence of a -1.5 MPa Ψ solute or matric stress. Error bars represent Stdev based on two independent experiments.

The induction of *csrA* and *hfq* genes suggests the propensity of small regulatory RNAs could mediate water stress responses.

Lastly, *turB* and *turE,* 2 of the 5 known H-NS like MvaT protein encoding genes, were expressed more during both water stress shock treatments (Table 2)(Renzi *et al.*, 2010). *TurD* (PP3693) expression increased by 1.8-fold following matric stress but did not meet our criterion for statistical significance based on p- (0.09) and q-values (0.016). MvaT homologs influence biofilm formation and the *mexEF* multidrug efflux pump operon, among multiple other genes in *P. aeruginosa* (Westfall *et al.*, 2006, Castang *et al.*, 2008). Previous *P. putida* KT2440 transcriptional profiling patterns and qRT-PCR analysis by Renzi *et. al.* (2010) found *turB* gene expression levels were most elevated during the early stationary phase whereas *turA* gene expression was greatest during early exponential phase. Considering *turB* transcripts levels increase upon matric and solute shock treatment, this is consistent with the hypothesis that select MvaT-like proteins have a role in water stress tolerance or that water stress shock leads to similar responses as cells in stationary phase.

Equilibration of intracellular water activity

Cells must respond quickly to sudden decreases in water availability by attempting to restore solute equilibrium and turgor pressure across the cell membrane using combinations of potassium ion uptake, closing mechanosensitive channels, and accumulating small, inert molecules known as compatible solutes (Imhoff, 1986, Landfald

and Strom, 1986, Lucht and Bremer, 1994, Kets *et al.*, 1996, Kempf and Bremer, 1998). Hydrophilin proteins also accumulate during water-limited conditions and are thought to be involved in water stress tolerance, although the exact mechanism is unclear (Battaglia *et al.*, 2008). Considering compatible solutes are the best understood aspect of water stress physiology little is known about the types of compatible solutes used during matric shock compared to the thermodynamically equivalent solute shock. During solute conditions *P. putida* accumulates the polyol-mannitol, N-acetylglutaminylglutamine amide (NAGGN) and betaine, but we do not know how quickly or to what extent the genes responsible for their accumulation responded to water stress shock (Kets *et al.*, 1996, Kets *et al.*, 1996).

We found, with the exception of NAGGN synthesis genes, compatible solute gene expression values are all within 25 % of one another in matric and solute shock conditions. Genes responsible for betaine accumulation were induced and include *betT*, *opuCA*-*D,* as well as *betA* and *aldH,* a *betB*-homology, for conversion of choline to betaine (Table 3) (Wargo *et al.*, 2008). Sufficient choline uptake and betaine accumulation correlate with abolishment of NAGGN synthesis in *S. meliloti* and *P. syringae* (Kets *et al.*, 1996, Talibart *et al.*, 1997, Kurz *et al.*, 2010). We observed induction of NAGGN synthesis genes *ggnA* and *ggnB* in both water stresses, although expression levels were greater during matric stress. As expected, since we used a defined growth medium, our data suggests there were insufficient amounts of choline betaine are present to be used as a compatible solute and, thus, cells require synthesis of an alternative compatible solute, such as NAGGN, trehalose, or mannitol.

Trehalose is generally present during NAGGN accumulation and we found the trehalose synthesis genes *treY, treZ, tres1* and *tres2* are induced (Table 3) (Kurz et al., 2010). Proteins encoded by the genes *treYZ* utilize malto-dextrin degradation as a source of trehalose, while the Tres1 and Tres2 proteins synthesizes trehalose from maltose (De Smet *et al.*, 2000, Kurz et al., 2010). *P. putida* likely relies on glycogen stores to produce malto-dextrins as a substrate for trehalose synthesis over that of maltose since the *treYZ* genes are more greatly induced than *treS1*. Gene expression of *treS2* was unaltered by water stress

compared to water-replete conditions. Additionally, *glgB*, a gene encoding a putative glycogen de-branching enzyme, is induced.

Together, greater gene induction values associated with trehalose synthesis genes, like NAGGN, during matric stress shock imply that they may accumulate more than in solute stress since cells likely have more negative effects from loss of water from the entire cell rather than just reduced intercellular water potential. Alternatively, non-reducing sugars, such as trehalose, can also help prevent lipid membrane distortions caused by anhydrobiosis through vitrification (Crowe et al., 1992). Some compatible solutes also have antioxidant capabilities that may be useful accumulation of reactive oxygen species (discussed below)(Smirnoff and Cumbes, 1989).

Table 3. Expression of compatible solute synthesis and transport genes

		Fold Induction	
Gene	Predicted Function	Solute	Matric
Betaine			
PP5064 *betA*	Choline dehydrogenase	1.6	1.3
PP3957 *betT*	High-affinity choline transport protein	1.8	2.4
PP5278 *aldH*	39% homology *to betB* betaine aldehyde dehydrogenase	15.7	18.1
PP0868 *opuCA*	Glycine betaine transport ATP-binding protein	14.2	17.3
PP0869 *opuCB*	Glycine betaine transport system permease	12.4	14.4
PP0870 *opuCC*	Substrate binding inner membrane domain component	27.1	29.0
PP0871 *opuCD*	Substrate binding inner membrane domain component	16.8	21.1
NAGGN			
PP1748	Putative glutamyl-aminopeptidase	5.5	11.6
PP1749 *ggnB*	Putative acetyltransferase; P. syringae *ggnB* homolog	5.3	10.7
PP1750 *ggnA*	Putative asparagine synthetase; P. syringae *ggnA* homolog	6.2	16.3
Trehalose			
PP4050 *glgA*	Glycogen synthesis	2.8	4.7
PP4051 *treZ*	Malto-oligotrehalose trehalohydrolase	2.0	2.3
PP4052 *malQ*	4-alpha-glucanotransferase	1.5	1.6
PP4053 *treY*	Malto-oligotrehalose synthase	1.3	1.6

Table 3. (continued)

		Fold Induction	
Gene	Predicted Function	Solute	Matric
PP4054 *treZ*	Malto-oligosyl trehalohydrolase	1.5	2.3
PP4058 *glgB*	Glycogen de-branching enzyme	1.4	1.8
PP4059 *treS2*	Trehalose synthase	1.4	1.6
PP2918 *treS1*	Trehalose synthase	1.2	1.8

Hydrophilins

P. putida contains 23 putative hydrophilins as identified by a genome-wide analysis of proteins that have high glycine content and a hydrophilicity value of 1.0 or greater (See Experimental Procedures). Gene expression values of three putative hydrophilins were 1.5-fold or greater during matric shock, with only one of these genes (PP4561) also induced by both water stresses (Table 4). This implies hydrophilins play a role, albeit limited, response to water stress shock. Originally discovered in plants, hydrophilins function to aid the cell cope with water-limiting conditions, especially in the presence of compatible solutes (Garay-Arroyo *et al.*, 2000, Reyes *et al.*, 2005, Battaglia *et al.*, 2008). Although how they function is unclear, some hydrophilins are predicted to interact with ribosomes and help stabilize ATPase (Garay-Arroyo et al., 2000). In *P. aeruginosa,* gene expression of three putative hydrophilin genes were increased by solute conditions; matric conditions were not tested (Aspedon et al., 2006). Interestingly, *D. radiodurans*
shifts its proteome to proportionally more hydrophilic proteins during dehydration which suggests an increased potential for hydrophilic proteins production, like hydrophilins (Krisko *et al.*, 2010).

Table 4. Putative hydrophilins and their respective microarray gene expression values

		Protein Properties			Fold Change in Expression [a]		Statistical Values	
Gene	Predicated Function	# Amino Acids	% Glycine	Hydro-Philicity [b]	Solute	Matric	p-	q-
PP0009	50S ribosomal protein	44	6.8	1.2	1.3	1.0	0.47	0.31
PP0023	hypothetical protein	42	7.1	2.4	-2.0	-2.2	0.06	0.14
PP0032	hypothetical protein	73	8.2	1.1	-1.1	-1.3	0.06	0.14
PP0067	lipoprotein, putative	118	9.3	1.2	-1.2	-1.6	0.48	0.31
PP1114	SEC-C domain protein	65	12.3	1.6	1.2	1.3	0.22	0.22
PP1561	phage holin, putative	122	8.2	1.2	-1.1	1.2	0.31	0.25
PP1568	hypothetical protein	74	9.5	1.2	1.0	1.2	0.26	0.23
PP1704	hypothetical protein	41	12.2	1.3	1.4	1.4	0.16	0.19
PP1911	50S ribosomal protein	60	6.7	1.1	1.3	1.0	0.53	0.32
PP2197	hypothetical protein	105	6.7	1.0	1.0	1.7	0.31	0.25
PP2850	hypothetical protein	37	24.3	1.4	1.0	1.1	0.92	0.43
PP3240	hypothetical protein	54	7.4	1.6	1.5	1.4	0.35	0.27
PP3269	hypothetical protein	85	15.3	1.6	1.0	1.1	0.52	0.32
PP3307	hypothetical protein	126	8.7	1.5	1.0	1.1	0.33	0.26
PP3883	phage holin, putative	106	7.6	1.0	-1.1	-1.2	0.01	0.07
PP3936	hypothetical protein	47	10.6	1.4	-1.1	-1.3	0.1	0.16
PP3938	hypothetical protein	44	11.4	1.1	1.0	1.0	0.58	0.33
PP3963	hypothetical protein	108	19.4	1.7	1.3	1.9	0.04	0.11
PP4365	flagellar biosynthesis chaperone	150	6.0	1.0	-1.1	-1.1	0.29	0.25
PP4561	CsbD family protein	59	13.6	1.0	6.0	10.5	0.0002	0.03
PP4640	hypothetical protein	65	10.8	1.0	1.1	1.2	0.18	0.2
PP4693	C4-type zinc finger	148	6.8	1.0	1.2	1.5	0.03	0.11
PP5049	hypothetical protein	35	22.9	1.3	1.0	1.0	0.99	0.44

[a] Fold differential gene expression during an -1.0 MPa Ψ solute or matric stress shock relative to water replete conditions

[b] Calculated hydrophilicity values based on ProtScale (www.ExPasy.org)

Protein fate

Numerous genes encoding chaperones and proteases, as well as two peptidyl-prolyl cis-trans isomerases are induced during water stress and are important for proper protein folding or degradation. Many of these genes were induced more by matric, than solute, shock, such as genes encoding the Lon and Clp-family proteases, GroEL, GroES and the peptidyl-prolyl cis-trans isomerases FklB-2 protein (Table 5). We also noted *algY, algU,* and *rpoH* gene expression is induced (Table 2 and 5). AlgY activity is a part of the envelope stress response that is activated upon accumulation of misfolded periplasmic proteins. AlgY

stimulates the release of the AlgU sigma factor into the cytosol which then induces *rpoH* gene expression. RpoH (σ^H) then coordinates the expression of multiple heat shock. We found induction in This is consistent with previous evidence showing moderate water-limitations place additional barriers against the ability of proteins to fold correctly and the need to facilitate proper protein folding, repair damaged proteins, or degrade unsalvageable proteins, all of which has been reviewed by Potts, 1999. Our findings are also in agreement with a previous Tn5-phoA fusion study in which many of these same genes were up-regulated by growth in matric conditions, although each one was not independently necessary for desiccation tolerance since single heat shock protein mutants were as sensitive to desiccation as wild-type cells (van de Mortel and Halverson, 2004). Together, these data imply matric stress cells elicit the envelope stress response leading to activation of the heat shock response necessary to maintain proper down-stream enzymatic functions.

Table 5. Expression of chaperone and proteases genes

		Fold Induction	
Gene	Predicted function	Solute	Matric
PP0252 *hslO*	33 kDa chaperonin	1.5	2.8
PP0625 *clpB*	ClpB protein	1.5	5.4
PP1360 *groES*	10 kDa chaperonin	6.0	10.3
PP1361 *groEL*	60 kDa chaperonin	6.6	13.1
PP1430 *algY*	Protease Do	1.9	2.7
PP1443 *lon-1*	ATP-dependent protease La	1.5	4.1
PP1714 *fklB-2*	Peptidyl-prolyl cis-trans isomerase	1.9	4.2
PP1719 *prc*	Periplasmic carboxy-terminal protease	1.4	2.6
PP1982 *ibpA*	16 kDa heat shock protein B	3.8	10.3
PP2302 *lon-2*	ATP-dependent protease La	1.9	5.5
PP2491	PfpI family, ATP-independent intracellular protease	1.3	6.3
PP4008 *clpA*	ATP-dependent clp protease ATP-binding subunit clpA	1.5	2.6
PP4009 *clpS*	ClpS, ATP-dependent Clp protease adaptor protein	1.8	3.5
PP4179 *htpG*	Chaperone protein htpG	4.8	14.4
PP4541 *ppiA*	Peptidyl-prolyl cis-trans isomerase A precursor	2.4	3.1
PP4726 *dnaJ*	Chaperone protein dnaJ	1.8	3.5
PP4727 *dnaK*	Chaperone protein dnaK	5.3	15.2
PP4728 *grpE*	Heat shock protein GrpE protein	8.1	20.7
PP4848	DnaJ family protein	1.1	1.7
PP5000 *hslV*	ATP-dependent protease hslV	3.9	11.3
PP5001 *hslU*	ATP-dependent hsl protease ATP-binding subunit hslU	2.9	10.8

Glutamine synthetases

P. putida contains one *glnA* glutamine synthetase gene, based on having the highest homology and similar genetic architecture surrounding the gene as other characterized *glnA* genes, and seven other homologous genes according to www.tigr.org annotation. Expression of g*lnA* and the PP5183 putative glutamine synthetase gene was repressed while all other putative glutamine synthetases genes are either induced or unchanged relative to water-replete conditions (Table 6). Gene PP5184, referred to as *pglS* for putative glutamine synthetase, was selected for qRT-PCR analysis. We found that during matric growth conditions, *pglS* expression in biofilm cells increased with time, although during solute stress there was an initial slight decrease in expression at 6 h (Fig. 6). Repression of *glnA* and PP5184 may be due their promoter regions containing predicated RpoN-binding sites, whereas the other putative glutamine synthetase genes do not (Cases et al., 2003). Furthermore, we can speculate that the concentration of RpoN is likely decreased given gene expression encoding other ECF sigma factor are increased. Also, as discussed earlier, other RpoN-regulated genes affecting glutamate/glutamine pool sizes are repressed such as the glutamate transporter genes PP1071-1068 and PP5234, a regulator of glutamine synthetase activity (Table 2). Presumably, the net effects of these changes are increased cellular glutamine concentrations implying that there are alternative routes for glutamine production during conditions that reduced *rpoN* gene expression.

Table 6. Expression of *glnA* and putative glutamine synthetase genes

		Fold Expression		Statistical Values	
Gene	Function	Solute	Matric	p-	q-
PP2178	Putative glutamine synthetase	1.1	1.1	0.86	0.40
PP3148	Putative glutamine synthetase	1.3	5.7	0.09	0.15
PP4399	Putative glutamine synthetase	1.2	1.2	0.16	0.19
PP4547	Putative glutamine synthetase	1.0	1.1	0.73	0.38
PP5046 *glnA*	Glutamine synthetase	-5.3	-4.7	0.004	0.053
PP5183	Putative glutamine synthetase	1.5	1.4	0.38	0.28
PP5184 *pglS*	Putative glutamine synthetase	3.4	5.7	0.007	0.062
PP5299	Putative glutamine synthetase	4.1	2.5	0.010	0.070

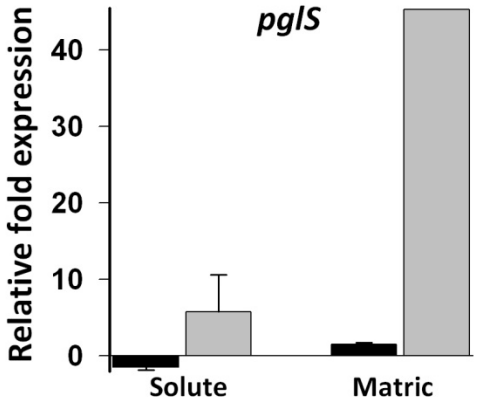

Figure 6. Q-RTPCR of the *pglS* gene from 6 h (black) and 24 h-old (gray) biofilm cells growing in the presence of a -1.5 MPa Ψ solute or matric stress. Error bars represent Stdev based on two independent experiments.

Oxidative Stress and ROS driven metabolic gene expression changes

Expression levels of several genes implicated in attenuation of reactive oxygen species (ROS) accumulation or coping with its damaging effects, were altered by matric, and to a lesser extent solute, stress (Table 7). There are at least four responses for dealing with ROS accumulation: antioxidant accumulation, using ROS resistant enzymes, repairing, replacing, or removing ROS damaged components through heat-shock proteins, and redirecting metabolic activities to prevent further ROS production. Our data supports all of these oxidative stress response mechanisms could be used and these responses are more evident during matric stress. This implies matric shocked cells combat more ROS promoted cell damage than solute-stressed cells (Table 7).

One gene, *bfr* (PP4856), annotated as a putative iron-scavenging bacterioferritin gene, is induced by water stress shock. *P. putida* contains two other bacterioferritin genes, *bfr*α and *bfr*β, but their expression was unchanged relative to water-replete conditions. This is consistent with the suggestion that Bfr is more similar to a Dps-protein rather than the other two known bacterioferritin proteins (Chen *et al.*, 2010). Dps is a non-specific DNA binding protein used in the defense against hydrogen peroxide and its expression is influenced by RpoS, the OxyR transcriptional activator, and IHF histone-like protein (Altuvia

et al., 1994). Biofilm cells grown in matric-stressed conditions for 6 and 24-h had continually elevated *bfr* gene expression compared to solute or water-replete grown cells. However, an induction value cannot be assigned since *bfr* transcripts isolated from solute and water-replete grown biofilm cells were below detectable qRT-PCR levels (data not shown).

Surprisingly, we found only a few genes associated with antioxidant production were induced. We noted the *gshA* gene, a glutamate-cysteine ligase likely involved in glutathione (L-γ-glutamyl-L-cysteinglycine) synthesis, was induced during water stress (Table 7). This agrees with previous data showing growth during matric stress induces expression of a glutamyl-transpeptidase gene responsible for transferring a glutamyl moiety to amino acids and the formation of glutathione. Had we extended our microarray studies beyond a 15 minute shock treatment we would anticipate finding greater induction of genes encoding antioxidants and the glutamyl-transpeptidase genes since previously we detected more ROS accumulation in aging biofilm cells grown under matric than solute stress conditions (Chang *et al.*, 2009). In addition to glutathione, there are additional compounds that may serve as an antioxidant, however, some of these, such as α-ketogluturate are pre-existing metabolic intermediates whose function cannot be assessed by our water stress shock results (Mailloux et al., 2009).

Gene expression of the two glucose-6-phosphate genes (*zwf1* and *zwf2*) and the 6-phosphogluconate dehydrogenase (*gnd*) gene were increased (Table 7). Zwf and Gnd protein activities generate NADPH and provide the reducing power for antioxidants during oxidative stress. NADPH production is especially useful since NADH production leads to downstream ROS generation during aerobic conditions through use of the electron transport chain complexes I, III, and IV (Singh *et al.*, 2008). Since sufficient membrane stress occurs during matric stress we anticipate enzymes located within the membrane may be damaged, such as those involved in the electron transport chain. Indeed, we noted reduced expression of many genes encoding various complex II electron-transport chain proteins, including *sdhA* during both water shock treatments. Although reduced expression of *sdhA*

seems counterintuitive since the cell would likely want to replace a damaged protein to retain its function, this may be not be the case if the function of the protein is not necessary. This agrees with the observation that many fermentative genes, such as alcohol and butanol dehydrogenase genes are induced (see Appendix D) and the arginine deminase pathway (discussed below) suggests a shift to an anoxic lifestyle during water stress shock. Indeed, a build-up in succinate pools caused by reduced succinate dehydrogenase activity, is suggested to elicit ROS responses and signal hypoxia (Selak *et al.*, 2005, MacKenzie *et al.*, 2007, Mailloux *et al.*, 2007). However, this shift is likely only transient since *sdhA* expression is equal to water replete condition in 6 and 24-h old biofilm cells grown in the presence of water stress (data not shown).

Table 7. Expression of oxidative responsive genes

		Fold Expression	
Gene name [a]	Predicted function [a]	Solute	Matric
Antioxidant production and ROS scavenging			
PP0235 *ahpC*	Peroxidredoxin	2.0	7.1
PP0115 *katE*	Catalse	1.4	2.4
PP0243 *gshA*	Glutathione biosynthesis; glutamate-cysteine ligase	1.6	2.3
PP0089 *osmC*	Osmotically activated protein; peroxidase activity		
PP2474	Glutathione S-transferase	1.7	3.0
PP1210	Ferroxidase/DNA-binding stress protein, putative	1.2	4.1
PP4856 *bfr*	Dps/ bacteriofferitin protein	6.7	15.1
TCA cycle and downstream metabolic enzyme alterations			
PP1755 *fumC-2*	Fumurate hydratase C	1.6	1.8
PP2112 *acnA*	Aconitate hydratase	1.6	2.4
PP4193 *sdhC*	Succinate dehydrogenase cytochrome b554	-1.6	-2.2
PP4192 *sdhD*	Succinate dehydrogenase membrane anchor	-1.7	-2.0
PP4190 *sdhB*	Succinate dehydrogenase iron-sulfer protein[c]	-1.3	-1.4
PP4191 *sdhA*	Succinate dehydrogenase flavoprotein[c]	-1.7	-2.0
PP0214 *gabT*	4-aminobutyrate aminotransferase	3.0	2.7
NADPH generating enzymes			
PP4042 *zwf-2*	Glucose-6-phosphate	1.8	3.2
PP1022 *zwf-1*	Glucose-6-phosphate	1.3	1.8
PP4043 *gnd*	6-phosphogluconate dehydrogenase	1.8	3.3
PP0215 *gabD*	Succinate semialdehyde dehydrogenase	2.6	2.3

[a] Gene name and predicted functions according to www.pseudomonas.org

Arginine Deiminase pathway

Expression of the PP0099-PP1002 genes encoding the arginine deiminase (ADI) pathway enzymes, are exclusively induced by matric shock (Table 8). This suggests that matric shock leads to either a decrease in cellular pH that is modulated by arginine deimination or an anoxic state eliciting the activity of the ADI pathway (Mercenier *et al.*, 1980, Poolman *et al.*, 1987). We explored the role of this pathway during biofilm formation under matric and solute stress conditions by qRT-PCR of *arcA* (PP1001) gene expression. Our findings show *arcA* gene expression is repressed during solute stress whereas it is induced only slightly during matric stress conditions (Table 8). Collectively, this suggests the ADI pathway is important during the initial, and to a lesser extent prolonged, exposure to matric stress conditions.

Table 8. Expression of Arginine Deiminase (ADI) pathway genes

			Fold Change in Expression					
			Solute			Matric		
			Time (h)			Time (h)		
PP #	Gene	Function	0.25	6	24	0.25	6	24
0999	*arcC*	Carbamate	1.3	-[a]	-	5.3	-	-
100	*argI*	Ornithine	1.3	-	-	10.1	-	-
100	*arcA*	Arginine	1.4	-2.6 ± 0.1[b]	-3.9 ± 1.0	10.4	1.9 ± 0.1	2.2 ± 0.9
100	*arc*	Arginine/ornithine	1.0	-	-	3.7	-	-

[a] (-) no data collected

[b] Average fold gene expression change ± stdev of two independent experiments

Motility

Genes associated with motility functions are repressed during water stress, although only significantly so by matric stress. These include genes encoding the flagellar structural proteins (*flgB, flgD, flgG, flgE, fliQ*) and the MotA protein (data not shown). These findings are consistent with previous evidence that hyperosmotic conditions reduce flagellar abundance in *E. coli* (Li *et al.*, 1993). Also, motility and exopolysaccharide gene expression are inversely related suggesting that an increase in EPS gene expression should decrease motility gene expression, as found during our study (Garrett *et al.*, 1999, Bahlawane *et al.*,

2008). Our findings are also congruent with the fact matric stress conditions imposes physical restraints on motility due to thinning of the water layer surrounding the cell and, therefore, expression of motility genes may become unnecessary (Howie *et al.*, 1987, van de Mortel and Halverson, 2004).

Mobile elements

The most repressed class of genes following water stress shocks were mobile elements, including putative group II intron maturases and IS element transposases. By relaxing our significance criterion we found eight out of the nine group II intron maturases identified in the KT2440 genome were repressed 1.5 to 3.2 fold by matric stress, and 1.4 to 1.9 fold by solute stress (Table 9). Group II intron maturases stabilize group II intron ribozymes which catalyze their own excision from precursor RNAs and subsequently integrate into DNA target sites by reverse transcription (Matsuura *et al.*, 2001, Lambowitz and Zimmerly, 2004). The KT2440 genome contains 41 IS elements, a very high number in comparison to other *P. putida* strains (Wu *et al.*, 2010). The majority of KT2440 IS elements in KT2440 are present in multiple copies (Nelson et al., 2002, Wu et al., 2010). Surprisingly, expression of at least one transposase gene present in 64 % of the IS elements were repressed, including nearly all copies of the ISPpu15, ISPpu14, ISPpu10 and ISPpu8 elements (Table 9). Numerous other IS elements transposase genes could be considered repressed if we relax the p- and q- scores that we deemed significant (Table 9). The targets of these IS elements are unclear, however, ISPpu10 is shown to associate with DNA repetitive extragenic palindromes (REP) sequences (Ramos-Gonzalez *et al.*, 2006) and one transposase gene, PP5290, is involved in maize seed attachment (Espinosa-Urgel 2000). Gene expression of a DNA site-specific recombinase and a Tn7 transposition gene are also repressed (Table 9).

Table 9. Mobile element genes repressed by either solute or matric shock.

		Fold Repression		Statistical Values	
Gene	Predicted gene function	Solute	Matric	p-	q-
PP0635	Group II intron-encoding maturase	1.4	1.9	0.06	0.13
PP1250	Group II intron-encoding maturase	1.9	2.8	0.05	0.12
PP1252	Group II intron-encoding maturase	1.9	3.2	0.03	0.11
PP1624	Group II intron-encoding maturase	1.5[a]	2.7	0.03	0.10
PP1846	Group II intron-encoding maturase	1.8	2.7	0.04	0.11
PP3172	Group II intron-encoding maturase	1.6	3.0	0.01	0.07
PP3820	Group II intron-encoding maturase	1.6	2.9	0.01	0.07
PP3868	Group II intron-encoding maturase	1.6	2.2	0.04	0.11
PP4409	Site-specific recombinase, phage	1.4	1.6	0.00	0.05
PP5405	Transposon Tn7 transposition protein	1.3	1.6	0.01	0.07
PP1865	ISPpu8, transposase	1.5	1.7	0.08	0.15
PP2114	ISPpu8, transposase	1.5	1.7	0.01	0.07
PP2218	ISPpu8, transposase	1.6	1.9	0.01	0.06
PP2522	ISPpu8, transposase	1.4	1.5	0.01	0.06
PP4318	ISPpu8, transposase	1.6	2.0	0.01	0.06
PP1133	ISPpu9, transposase	1.8	2.2	0.18	0.20
PP1260	ISPpu9, transposase	1.3	2.4	0.07	0.14
PP2570	ISPpu9, transposase	1.4	2.0	0.15	0.18
PP3381	ISPpu9, transposase	1.6	1.8	0.37	0.27
PP3586	ISPpu9, transposase	1.4	2.3	0.06	0.14
PP4603	ISPpu9, transposase	1.6	2.6	0.13	0.17
PP4791	ISPpu9, transposase	1.4	1.8	0.19	0.20
PP0526	ISPpu10, transposase	1.7	2.9	0.10	0.16
PP1653	ISPpu10, transposase	2.0	3.3	0.06	0.13
PP2134	ISPpu10, transposase	2.2	3.9	0.01	0.07
PP3502	ISPpu10, transposase	2.2	3.3	0.06	0.13
PP4599	ISPpu10, transposase	2.0	3.3	0.06	0.13
PP5050	ISPpu10, transposase	1.7	2.8	0.06	0.13
PP5290	ISPpu10, transposase	1.9	2.9	0.03	0.10
PP3986	ISPpu13, transposase Orf1	1.0	1.6	0.27	0.24
PP3966	ISPpu14, transposase Orf1	2.2	4.4	0.00	0.05
PP3979	ISPpu14, transposase Orf1	2.0	3.8	0.01	0.06
PP3499	ISPpu14, transposase Orf1	2.0	2.9	0.01	0.08
PP4437	ISPpu14, transposase Orf1	2.1	3.6	0.01	0.06

[a]Shaded cells meet the criteria set for significant based previously defined p- and q-value; unshaded cells either were below the 1.5-fold cutoff or were repressed 1.5 or greater but had higher than allowable p- and q- values set for signifying significant gene expressional changes.

Table 9. (continued)

		Fold Repression		Statistical Values	
Gene	Predicted gene function	Solute	Matric	p-	q-
PP4441	ISPpu14, transposase Orf1	2.1	3.2	0.01	0.07
PP5398	ISPpu14, transposase Orf1	2.2	3.1	0.01	0.06
PP0638	ISPpu15, transposase Orf1	2.3	6.7	0.01	0.06
PP4024	ISPpu15, transposase Orf1	2.5	6.9	0.01	0.06
PP4092	ISPpu15, transposase Orf1	2.3	6.7	0.01	0.06
PP4746	ISPpu15, transposase Orf1	2.1	6.4	0.01	0.07

Concluding remarks

The sheer number of genes regulated by both matric and solute stresses, and the even greater number regulated solely by a matric stress, suggest that adaptation to matric stress is more complex than to the thermodynamically equivalent solute stress, although some overlap exists. The signals activating transcription of these genes remain to be determined, although at some level cell, protein, or membrane hydration probably plays a role. It is clear that based on these and previously published findings, we suggest that several regulatory networks directly or indirectly control the expression of these genes. In particular, our data suggests that activation of an envelope stress response would counter the detrimental effects of matric stress on protein folding and illustrates the importance of maintaining a functional cell envelope. As we identified numerous differentially expressed genes of unknown function, we may be uncovering novel aspects of bacterial physiology required for growth and survival in low-water-content habitats which are distinct from those for survival in high osmolarity habitats.

Experimental procedures

Bacterial strains, growth conditions, and culture treatments

Pseudomonas putida KT2440 was grown on King's B (KB) medium supplemented with 50µg/mL rifampin for 48 h at 28 °C (King *et al.*, 1954). Single colonies were inoculated into ½ 21C medium containing 0.3 % glucose and 5mM succinate (½ 21C-GS) and grown until late stationary phase at 200 rpm, 28°C. A 10 µL aliquot was subcultured into 30 mL fresh ½ 21C-GS and grown to an optical density (OD_{600}) of approx. 0.3, corresponding to 10^8

cells mL^{-1}. Cells were centrifuged and suspended in 3.6 mL pre-warmed ½ 21C-GS to an OD_{600} of 0.4. 300 µL aliquots were then transferred to centrifuge tubes and 0.8 mL ½ 21C-GS medium containing NaCl, Peg8000, or no amendments were added to create a 1.0 MPa decrease in water potential or water replete conditions, respectively. Cells were incubated shaking at 28° C for 15 minutes before addition of RNA Protect (Qiagen) to stabilize RNA and prevent further transcript production. RNA was either isolated from cells immediately or cell pellets stored for up to two weeks prior to RNA harvest, as per manufactures instructions.

RNA harvest and microarray design

RNA was purified from a total of four samples using the RNeasy mini kit (Qiagen). Contaminating DNA was digested using both on-column DNase digestion and Turbo DNase (Ambion). RNA samples were pooled so that each sample contained two replicates isolated on separate days. RNA quantity and integrity was measured using the Nanodrop 2000 (Thermo Scientific) and the Agilent 2100 Bioanalyzer, respectfully, prior to submission of samples to NimbleGen (Madison, WI) for downstream microarray processing. All DNA sequence probes were based on the genome sequenced *P. putida* strain KT2440 (Nelson et al., 2002).

Data Analysis

Robust multiarray averaging (RMA) based on background corrected probe fluorescent intensity values were done to allow comparisons between microarray datasets. RMA calculations included quantile normalization to remove non-biological variation and median polishing. An estimated mean for each microarray spot representing a gene was found and resulting values were averaged between replicates. Gene expression was considered significantly altered if transcript abundance was at least 1.5-fold different from water-replete conditions with a p-value ≤ 0.05 and a false discovery rate (q- value) ≤ 0.10.

Quantitative RT-PCR

P. putida mt2 biofilms cells cultivated on ½ 21C-GS solid media were suspended into ½ 21C-GS medium to an OD_{600} nm of 0.1. From this cell suspension, a 100 μL aliquot was plated directly (6 h) or diluted 1:1000 (24 and 48 h) onto nylon membranes overlaying solid ½ 21C-GS plates. Plates were incubated at 28 C in airtight containers. Media was amended with NaCl or PEG8000 to lower the water potential by 1.5 MPa. For RNA isolation, nylon membranes were removed and vortexed in a 50 mL Falcon tube containing 10 mL of 1:2 part solution of RNA Protect (Qiagen): ½ 21C-GS broth. RNA was isolated and quantified as described above. Q-RTPCR was performed on either a iCycler or DNA Opticon 2 (BioRad) using Quanta 1-step sybr green kit (VWR), 5 pmol/uL primers (Table 11), and the following program: 50 °C 10 min, 94 °C 5 min, (94 °C 15 sec, 60 °C 30 sec, read) x40, 72 °C 3 min, 4 °C hold. Resulting changes in Ct values were expressed as gene fold induction or repression relative to unamended (water-replete) treatments and *rimM* gene Ct values, using the ΔΔCt method (Livak and Schmittgen, 2001).

Table 11. qRT-PCR primers used in this study

Gene	PP#	Forward primer	Reverse Primer
rimM	1463	GAGCGGTTACGAAATCTGCATCCC	CATTACATCGTTCGCACCGGTCTC
alg8	1287	ATGATCTTCCTCTACGGCGTGTACC	AGCAGTTCATCGGACATTTCCACC
mucA	1428	TGAAGCTTTGCAGGAATCGCTGTC	TTAGGCAGCAGCAGTTCCTTGTG
algU	1427	ACGGGCTGAGTTACGAAGACATTG	AACGGCTGCAGGGCTTTATCTATG
nvdB	1740	GCCAAGGCGGTACTGTATGGTTTC	CACATAAGGCAACGACTGCACCAG
opgG	5026	CTCACCGGGCGTGACAAATACAAG	GTGTCGCTGAGCACAAAGAAGTCG
oprH	1185	TGCTTGGCAGCTACGACTTGTTC	TAGGCATAACCGTAGTCAGTGTCC
sdhC	4193	AGCCAACGACCTGTAAACCTAGAC	CGACTTGCCCAGTGCATAAAGCAT
arcC	1001	TCCCGGAAGTGGTGAAAGAGATTG	TGTTGATGCGGCGTACATCCATTC
bfnA	4856	AGTTTCTGGAACATGCCAACCAGG	TCCAGCACCATCTCTTTGAGCGAA
pglS	5184	TATGAATGGTGTGGTGCGTGGCAA	AACCGTTGATGTCCAGGGCAAAGA

Identification of putative hydrophilins

To identify putative hydrophilins *P. putida* predicted protein sequence encoded by all genes was imported from www.pseudomonas.com into a Microsoft Excel spreadsheet. The excel LEN function was used to count the number of amino acid residues in each protein and

those with 150 or fewer amino acids were parsed into their individual amino acid components to survey for the proportion of glycine residues using Excel COUNTIF function. Proteins comprised of 6 % or more glycine residues were assigned hydrophilicity values using the ProtScale program (ExPasy.org). Proteins that had hydrophilicity values less than or equal to 1.0 were considered putative hydrophilins.

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Chapter 6. General discussion and future directions

General Discussion

The availability of water is an essential factor for driving bacterial activity and persistence in soil. Soils frequently undergo fluctuations in water availability. Water availability is measured in terms of its water potential that is influenced by the concentration of solutes (solute potential) or the physical sorption of water to surfaces (matric potential). Increasing solute and matric potentials both cause water activity in cells to decrease, however, in high solute conditions, cells are bathed in water of diminished activity whereas during matric stress conditions water is physically drawn out of the cell by membrane impermeable substances and any remaining water is bound by capillary or surface tension forces causing it to be unavailable for microbial use, thus dehydrating the cells. Matric stress conditions are dominant in low-water content environments, such as those occurring periodically in the soil, yet microbial responses to solute stress are better understood than thermodynamically equivalent matric stress.

It is no surprise, then, that bacterial responses to matric and solute stress are similar, but yet distinct. One means of maintaining hydration and reducing direct contact with the external environment is to form a biofilm. Biofilm exopolymeric matrix is comprised of a cornucopia of material including exopolysaccharides, lipids, DNA, and proteins. The ratio and types of these materials can change based on the metabolic status of the enmeshed cells and their response to changing environmental conditions. Until recently the hygroscopic exopolysaccharide, alginate, was the only characterized EPS in the *P. putida* mt2 biofilm matrix. We expanded on this by exploring the role of a novel EPS, Pea and a cellulose-like polymer (Bcs) on biofilm formation (Chapter 3). We found the cellulase-sensitive Pea EPS is essential for maintaining biofilm stability as both pellicles and adherence to glass coverslips was reduced in Δ*pea* biofilm cells. We also found Bcs has a role in maintaining the competitive colonization abilities of cells in the rhizosphere, while its role in biofilm formation and adherence to abiotic surfaces is more difficult to detect. Biofilm cells defective in Bcs or Pea production were unable to maintain biofilm biomass on

microtiter plate wells and biomass was decrease farther in cells lacking both polymers, suggesting that Bcs and Pea may both reinforce the matrix and may do so through interactions with one another. Lastly, biofilm cells unable to produce alginate and either Pea or Bcs produce greater amounts of total exopolysaccharides than wild-type or alginate-deficient mutants. This suggests that in the absence of alginate, Pea and Bcs may contribute to matric stress tolerance and that either exopolysaccharide likely compensates for the lack of the other. Indeed, alginate gene expression is a higher in EPS mutant biofilm cells suggesting the each EPS has a role in matric stress physiology.

Proteins are also a part of the *P. putida* biofilm matrix and the knowledge of the contribution and interactions between various proteins and EPS components on biofilm stability is growing. We add to this body of literature by describing the role of an amyloid-like protein, purlin, and in pellicle formation, biofilm attachment, and alginate gene expression (Chapter 3). Our current model of the composition of the biofilm matrix during water-limited conditions is not fully formed but our data suggest that purli likely interacts with both Bcs and Pea. This stems from the observations that in the absence of purli abiotic biofilm adherence is slightly reduced compared to wild-type cells, however absence of purli in Pea-deficient cells greatly reduces early pellicle formation and biofilm stability on microtiter plate, but not necessarily glass coverslips. Glass coverslip assays measuring the percentage of biofilm cells covering the air-liquid interface were highly variable within a single sample (Chapter 3, Table 2). This suggests that EPS and purli contribute to, but are not the only factors needed for *P. putida* biofilm formation. In fact, recent findings by Tim Tolker-Nielsen (personal communication) show that EPS is not required for biofilm formation and only are necessary for stabilization of the biofilm matrix. Alginate biosynthetic operon gene expression typically occurs in response to the induction of the envelope stress response during water-limiting conditions, whereas during water-replete conditions there is no need for the envelope stress response or alginate gene expression. In all cells alginate gene expression was elevated by matric stress whereas Δ*bcs*Δ*psgA* and Δ*bcs*Δ*pea*Δ*psgA* cells have high alginate gene expression under water replete and water

stress growth conditions. This suggests that in the absence of Bcs and Purli, the regulatory network or factors regulating the envelope stress response become uncoupled leading to greater alginate gene expression. Since the matric stress conditions causes cellular damage resulting in eliciting the envelope stress response pathway, which includes activation of the AlgU sigma factor needed for alginate gene expression, it is likely that the interaction of purli and EPS are decouple the envelope stress response network.

Phenotypic traits used to reduced or tolerate reduced water potentials are ineffective if the genes that regulate the traits cannot respond to changing environmental conditions. To understand which genes change their expression in response to lowered matric and solute water potentials we performed whole genome transcriptional profiling on *P. putida* cells exposed to a matric or a solute shock. While the expression of genes implicated in many, overlapping, response pathways were altered, exposure to thermodynamically equivalent matric stress shock caused greater induction or repression of genes than exposure to solute shock. Sudden water-limitations also caused *P. putida* cells to induce expression of many genes used during anaerobic fermentation suggesting that cells enter an anoxic state. Considering we also noted induction of genes used during the oxidative stress response switching to an anoxic-like metabolic state may be correlated with the need to reduce reactive oxygen species generation. Several genes encoding unknown proteins were uniquely induced by matric shock conditions implying that novel, uncharacterized responses of *P. putida* cells occur and may be pivotal to sustaining life in low-water content habitats.

Future directions

The studies in this dissertation provide key insights into the biofilm matrix and water stress physiology. However, these insights are far from complete. The recent discovery of a fourth exopolysaccharide, Peb, complicates the interpretation of our results since without understanding the interactive role of Peb in the biofilm matrix it becomes more difficult to attribute water stress response to any one EPS. Furthermore, further characterization of the chemical composition of Pea and Bcs would be useful. This may be challenging since,

for example, our best estimate is that Bcs comprises less than 0.25% of the exopolysaccharides recovered from P. putida biofilms. Knowing the chemical structure of Bcs and Pea would also allow us to understand further elucidate the genetic pathways involved in their synthesis. Lastly, to be able to associate a chemical or immunological test with each exopolysaccharide component would be useful in allowing us to directly measure and quantify the presence of a specific EPS in wild-type biofilms under various environments conditions. Antibodies conjugated to fluorescent dyes or naturally occurring EPS-specific fluorogenic dyes would be useful in elucidating the architectural association of EPS component with each other, with the cell, or within the biofilm using direct microscopic observations.

The contribution of purli to *P. putida* biofilms properties and desiccation tolerance need to be explored further. Specifically, we need to generate a *ΔpeaΔpsgA* pWspR19 strain to ascertain if pellicle formation is dependent on Pea and purli interactions or requires Bcs involvement. Gene expression of *psgA* is elevated during growth in water-limited conditions. We speculate that this is due to the additional support within a biofilm matrix during enhanced EPS production. Two lines of evidence would support this hypothesis: first, and the most direct, is isolation of extracellular proteins from *P. putida* biofilms during water-replete and water-limited conditions to probe the levels of purli synthesized using the WO1 antibody or a purli-specific antibody. If our hypothesis is correct we would anticipate more extracellular proteins isolated from biofilm cells grown in low-water content conditions would react with WO1-antibodies than those isolated from cells grown in water replete or high osmotic conditions. Secondly, comparison of *psgG* and *psgB* transcript levels via qRT-PCR would provide further indirect evidence for purli fiber accumulation during matric stress conditions since, in *E. coli,* the CsgG lipoprotein is required for *csgA* secretion and CsgB is required for nucleation of CsgA. Of course, the possibility also exists that increased purlin proteins may results in increased fiber length rather than increase in the total number of purli fibers; this distinction would only be possible through electron microscopy or immunological based fluorescence microscopy methods.

Initial cell attachment to a surface is mediated by curli and tafi fibers in *E. coli* and *Salmonella.* We presented preliminary evidence that purli fibers are also involved in the initial attachment of *P. putida* cells to an abiotic surface, although these experiments need to be repeated to validate our observation. Lastly, we have indirect evidence that purli interactions with Bcs may alters alginate gene expression. Alginate gene expression is one set of genes expressed as part of the envelope stress response. Lack of tafi, and amyloid fiber in *Salmonella,* and cellulose reduces biofilm formation and long-term cell survival during desiccation. Together, this suggests that tafi and cellulose contribute to desiccation tolerance and raises the possibility that Bcs and purli could do that same in *P. putida.* To test this possibility tolerance assays and individual cell survival counts need to be assessed during desiccation.

Lastly, results from our microarray data were intriguing. We anticipated that many of the significantly altered responses would be shared during solute and matric stress conditions but also anticipated genes responses solely to matric or solutes stress conditions as well. However, many genes encoding unknown proteins were uniquely induced by matric stress conditions aiding us to identify key genetic responses and, by inference, metabolic pathways that contribution to matric stress tolerance. One gene in particular, PP4856, annotated as a bacterioferritin-like gene, was induced by matric stress conditions but neither of the other not *P. putida* bacterioferritin genes, *bfr*α and *bfrβ,* were. In fact, PP4856 has been suggested to be more similar to Dps than to Bfr genes. Our lab posses the *bfr*α and *bfrβ* mutant strains and has a vector for constructing a *dps* mutant. A comparison between the survival rates of PP4856, *dps, bfr*α and *bfrβ* mutant strains to both desiccation and reactive oxygen species accumulation caused by paraquat treatment, may be beneficial to our understanding of the role of the PP4856 gene to matric stress tolerance. Lastly, to ascertain if the same genes that alter their expression in response to matric stress shock are also altered during growth in low water-content conditions further microarray studies comparing gene expression during longer exposure to matric and water-replete conditions is required.

Appendix A. Supplemental information for Chapter 3: Cell-cell and cell-surface interactions mediated by cellulose and a novel exopolysaccharide contribute to *Pseudomonas putida* biofilm formation and fitness under water-limiting conditions

Table A1. Primers used in study

Primer Name	Oligo sequence (5'-3')	Source or reference
2632SOE1F	AAGCTTTGGGTTACCTGTACCTACGCC	This Study
2633SOE2R	GAAGCAGCTCCAGCCTACACAATGCAAAGCCAGGCTGGTCAT	This Study
2636SOE5F	GGAGGATATTCATATGGACCATGGTAGGCAAGCTCGATGCGGTG	This Study
2637SOE6R	AAGCTTGGCTCAAGCAACTGCAAACGG	This Study
pKD4-F	CCATGGTCCATATGAATATCCTCC	This Study
pKD4-R	ATTGTGTAGGCTGGAGCTGCTTC	This Study
PP3138F	CGCACCAATGAACAGGCAGG	This Study
Km3138Up	GAAGCAGCTCCAGCCTACACAATCACTTCAGGCTGTCGACTACG	This Study
Km3141Dn	GGAGGATATTCATATGGACCATGGGCTGCTACAGTGATATCAATC GC	This Study
PP3143R2	CAGTTCGGAATATGCCGAGTG	This Study
CompPea-F	ATGCGCATTGCTTACTTCATC	This Study
CompPea-R2	TACAACAGGGTACGCAACAGG	This Study
BcsCompF	ATGACCAGCCTGGCTTTGCATG	This Study
BcsCompR	GCATCATCACCCCGCCTCAC	This Study
5-PP2634	TACGGGTACTGGTGGCAGAAG	This Study
PP2637R	TATGCTCACTGTTGCGTACCC	This Study
Alg8-RT2F	ATGATCTTCCTCTACGGCGTGTACC	(Li et al., 2010)
Alg8-RT2R	AGCAGTTCATCGGACATTTCCACC	(Li et al., 2010)
BcsA-RT1F	ATGTACGGGTACTGGTGGCAGAAG	This Study
BcsA-RT1R	CGGCAAGCGATCACGAAACAAGTC	This Study
3140-RT4F	GCGGCAAAGGTTACGCATTGGATTTC	This Study
3140-RT4R	TGCACGCATCAGATACAGCGAC	This Study
RimM-RT2F	GAGCGGTTACGAAATCTGCATCCC	(Li et al., 2010)
RimM-RT2R	CATTACATCGTTCGCACCGGTCTC	(Li et al., 2010)

Table A2. Competitive root colonization by mt2 and EPS-deficient mutants

	Proportion (%) ± SEM of each strain[a]					
	Δ*bcsQAB*		Δ*peaGHI*		Δ*bcsQAB*Δ*peaGHI*	
Experiment	Initial	Final	Initial	Final	Initial	Final
1	43.7	3.9 ± 2.2	37.8	48.7 ± 17.0	ND[b]	ND
2	61.1	41.0 ± 3.9	40.4	25.0 ± 4.5	67.1	30.9 ± 5.8
3	50.7	30.8 ± 4.0	12.0	44.5 ± 2.7	36.6	39.4 ± 3.8

[a] Initial proportion of mutant cells in the inoculum and the final proportion recovered from roots 7 days post-inoculation in direct competition with the wild type mt2

[b] ND, No data

Table A3. Percent *P. putida* pP*algD-gfp* and pP*nptII-gfp* cell populations expressing GFP during water replete growth conditions. Data is average and SEM of three independent experiments each with three replicates. *P. putida* mt2 (pP*nptII-gfp*) was not included in statistical analysis for significance.

Strain	% GFP expressing cells ± SEM	
mt2 (pP*algD-gfp*)	0.30 ± 0.19	A
Δ*peaGHI* (pP*algD-gfp*)	0.16 ± 0.07	B
Δ*bcsQAB* (pP*algD-gfp*)	0.14 ± 0.04	AB
Δ*bcs*Δ*pea* (pP*algD-gfp*)	0.08 ± 0.03	B
Δ*bcs*Δ*alg* (pP*algD-gfp*)	0.10 ± 0.06	AB
Δ*pea*Δ*alg* (pP*algD-gfp*)	0.23 ± 0.12	AB
Δ*algD* (pP*algD-gfp*)	0.18 ± 0.08	B
mt2 (pP*nptII-gfp*)	89.8 ± 4.7	

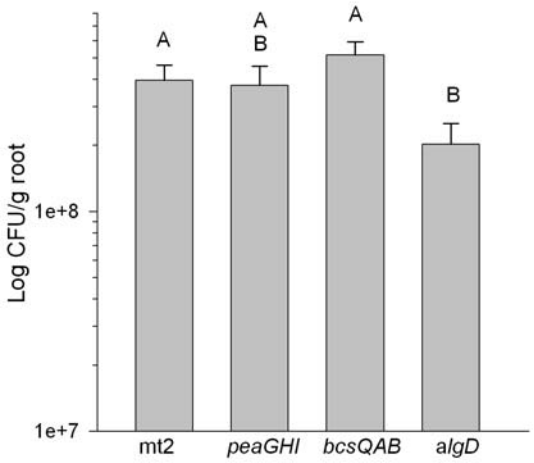

Figure A1. Maize root colonization assay of single *P. putida* strains. Log CFU/g maize root material colonized by *P. putida* strains in the absence of competition after 7 days

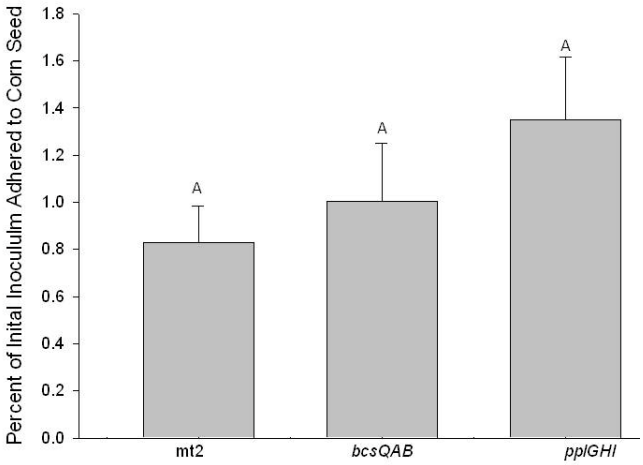

Figure A2. Corn seed adherence assay. Graph shows average and standard deviation of percentage of cell population adhering to corn seed after 1 hour relative to initial inoculum. Studies were repeated twice with each experiment containing 3 replicates and 2 subsamples of each replicate.

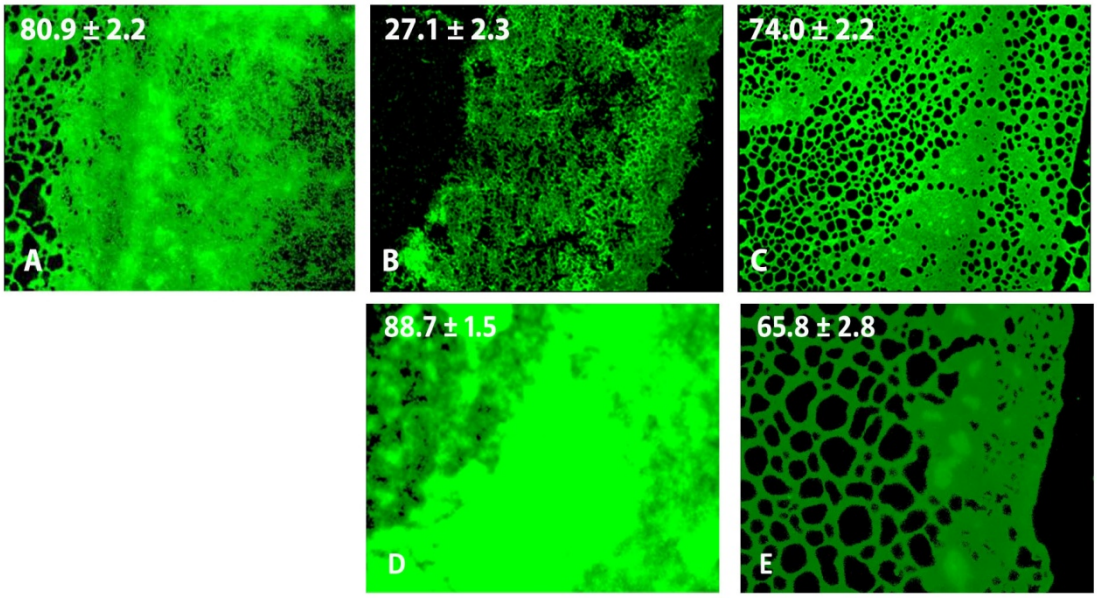

Figure A3. Complementation assays of biofilm formation on glass coverslips. Biofilm formation on glass coverslips 24 h post-inoculation by miniTn7gfp_{AAV} tagged LH1 (pME6041) (A), Δ*peaGHI* (pME6041) (B), Δ*peaGHI* (pMe-Pea) (C), Δ*bcsQAB*Δ*peaGHI* (pME6041) (D), and Δ*bcsQAB*Δ*peaGHI (*pMe-Bcs). Ten epifluorescence microscopy images were taken per replicate using a 10x objective. Values are the mean ± SEM % surface area coverage at the air-liquid interface of two to three experiments, each comprised of three replicates.

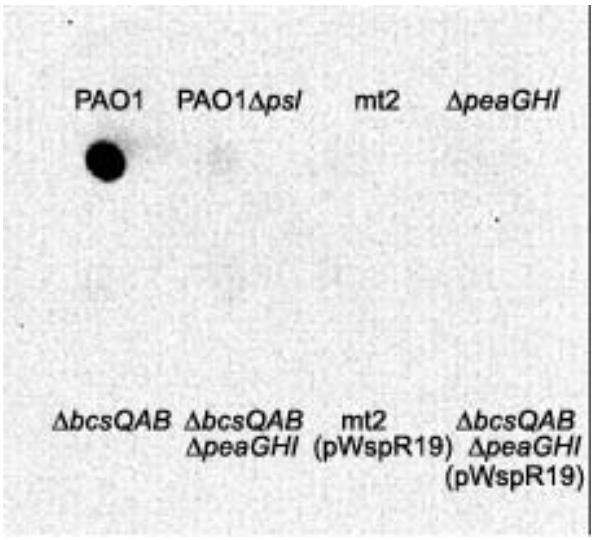

Figure A4. Reactivity of crude EPS from *P. aeruginosa* PA01 and *P. putida* mt2 with α-Psl antisera. Crude EPS isolated from *P. aeruginosa* PAO1 or *P. putida* mt2 and mutants were spotted onto nitrocellulose membranes and probed with rabbit anti-Psl primary antibody followed by secondary donkey anti-rabbit-HRP conjugated secondary antibody. Strain names appear above or below relative to the crude EPS spots in the top or bottom rows, respectively.

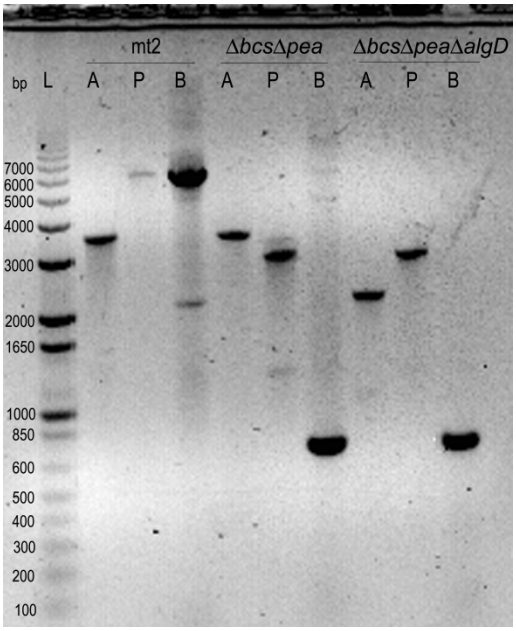

Figure A5. PCR verification of gene deletions in *P. putida* strains used in Chapter 3
Primer sets flanking *algD* (A), *peaGHI* (P), and *bcsQAB* (B) genes were used to verify *deletions* in Δ*bcs*Δ*pea* and Δ*bcs*Δ*pea*Δ*algD* mutants. Pea, Bcs, and algD amplification products were reduced from 6.2, 6.1, and 3.7 kb to 3.2, 0.75, and 2.2 kb, respectively.

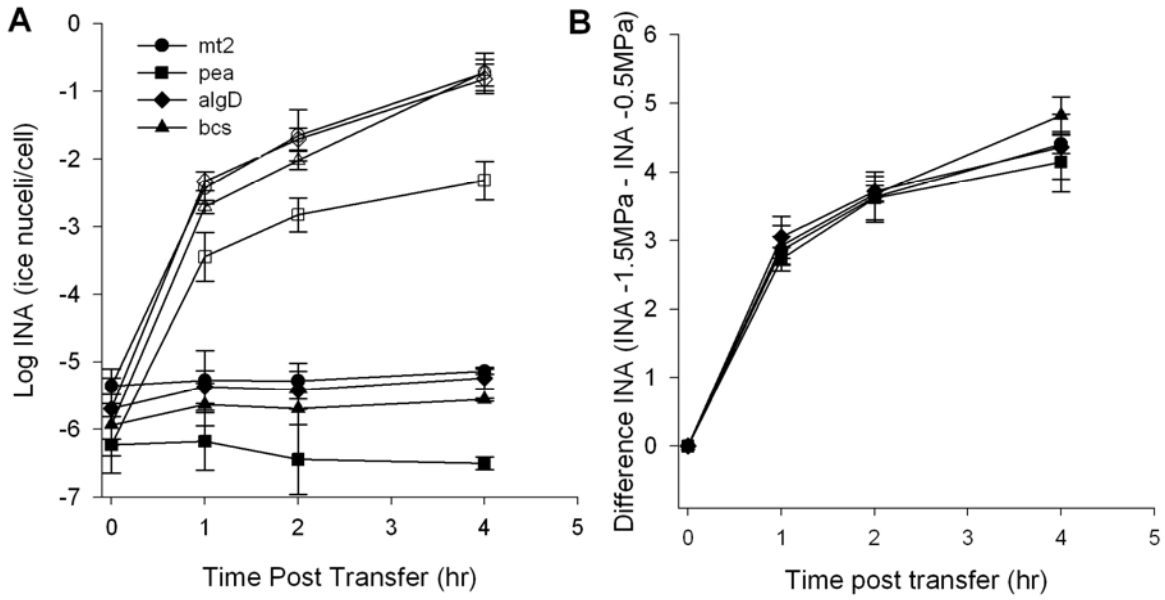

Figure A6. Dehydration shock assays of *P. putida* mt2 pP*proU-inaZ* and EPS-deficient pP*proU-inaZ* strains. Cells were grown on nylon membranes overlaying ½ TYE-H medium lowered to a water potential of 0.5 MPa with Peg8000 for 24 hours prior to transferring to either -1.5 MPa ψ or -0.5 MPa ψ. (A) Log INA activity per cell after hours post transfer and (B) Comparison of the number of ice nuclei per cell in cells continuously exposed to non-shock and dehydration shock conditions.

Appendix B. FAME analysis of EPS and Purli mutants

Table B1. Percentage 16:0 Fatty Acid Residues

Strain	Basal	Solute	Matric
mt2	27.5 ± 0.5 A B	29.8 ± 1.5 A	27.3 ± 0.6 B C
Δ*bcs*	25.9 ± 1.3 B C	29.5 ± 0.5 A	28.1 ± 0.1 A B
Δ*bcs*Δ*pea*	27.8 ± 0.1 C	30.1 ± 0.6 A	27.9 ± 0.1 A B
Δ*psgA*	29.0 ± 0.5 A	30.4 ± 0.4 A	28.6 ± 0.3 A
Δ*bcs*Δ*psgA*	28.2 ± 0.9 A	30.5 ± 0.3 A	28.3 ± 0.5 A B
Δ*bcs*Δ*pea*Δ*psgA*	25.8 ± 0.4 A	30.0 ± 1.7 A	26.0 ± 0.9 C

Table B2. Percentage 16:1cis Fatty Acid Residues

Strain	Basal	Solute	Matric
mt2	21.2 ± 0.8 B	20.8 ± 1.4 A	20.7 ± 3.1 A
Δ*bcs*	24.9 ± 1.9 A B	23 ± 1.6 A	23 ± 2.7 A
Δ*bcs*Δ*pea*	22.6 ± 1 A B	24.9 ± 0.4 A	21.8 ± 0.7 A
Δ*psgA*	25.8 ± 1.7 A	20.7 ± 1.5 A	23.1 ± 1.1 A
Δ*bcs*Δ*psgA*	24 ± 2 A B	24.6 ± 1.4 A	19.7 ± 0.6 A
Δ*bcs*Δ*pea*Δ*psgA*	23.7 ± 1.1 A B	24.7 ± 2.8 A	22.4 ± 0.4 A

Table B3. Percentage 16:1trans Fatty Acid Residues

Strain	Basal	Solute	Matric
mt2	0 ± 0 A	0.5 ± 0.7 A	0.6 ± 0.7 B
Δ*bcs*	0 ± 0 A	0 ± 0 A	0.6 ± 0.7 B
Δ*bcs*Δ*pea*	0 ± 0 A	0 ± 0 A	0 ± 0 B
Δ*psgA*	0 ± 0 A	2.6 ± 0.2 B	1.7 ± 1.7 A B
Δ*bcs*Δ*psgA*	0 ± 0 A	0 ± 0 A	2.6 ± 0.2 A
Δ*bcs*Δ*pea*Δ*psgA*	1.0 ± 1.2 A	0 ± 0 A	0 ± 0 B

Table B4. Percentage Cyclo17 Acid Residues

Strain	Basal	Solute	Matric
mt2	14.5 ± 1.9 A	14.5 ± 1.4 A	13.2 ± 2.2 A
Δ*bcs*	11.4 ± 0.9 A	13.1 ± 1.9 A	10.1 ± 2 A
Δ*bcs*Δ*pea*	12.5 ± 1.6 A	10.6 ± 0.3 A	12.1 ± 0.7 A
Δ*psgA*	13.8 ± 0.9 A	13.8 ± 2.5 A	11.9 ± 0.9 A
Δ*bcs*Δ*psgA*	12.8 ± 2.6 A	12 ± 0.2 A	12 ± 0.4 A
Δ*bcs*Δ*pea*Δ*psgA*	12.6 ± 0.3 A	11.9 ± 3.3 A	13.4 ± 0.1 A

Table B5. Ratio Saturated to Unsaturated Fatty Acids
(C16+Cyclo17)/(C16:1cis + C16:1trans)

Strain	Basal		Solute		Matric	
mt2	2.0 ± 0.1	A	2.1 ± 0.2	A	2.0 ± 0.3	A
Δ*bcs*	1.5 ± 0.2	B	1.9 ± 0.2	A	1.6 ± 0.2	A
Δ*bcs*Δ*pea*	1.8 ± 0.1	A B	1.6 ± 0.04	A	1.8 ± 0.1	A
Δ*psgA*	1.7 ± 0.2	A B	1.9 ± 0.2	A	1.7 ± 0.1	A
Δ*bcs*Δ*psgA*	1.7 ± 0.2	A B	1.7 ± 0.1	A	1.8 ± 0.02	A
Δ*bcs*Δ*pea*Δ*psgA*	1.6 ± 0.1	B	1.7 ± 0.3	A	1.8 ± 0.04	A

Table B6. Ratio Saturated to Unsaturated Fatty Acids
(C16+ C18+ Cyclo17)/(C18:1+ C16:1cis + C16:1trans)

Strain	Basal		Solute		Matric	
mt2	2.9 ± 0.2	A	2.9 ± 0.3	A	2.9 ± 0.4	A
Δ*bcs*	2.3 ± 0.3	B	2.7 ± 0.2	A	2.5 ± 0.3	A
Δ*bcs*Δ*pea*	2.7 ± 0.2	A B	2.4 ± 0.1	A	2.7 ± 0.1	A
Δ*psgA*	2.3 ± 0.2	B	2.7 ± 0.3	A	2.4 ± 0.1	A
Δ*bcs*Δ*psgA*	2.5 ± 0.2	A B	2.5 ± 0.1	A	2.7 ± 0.04	A
Δ*bcs*Δ*pea*Δ*psgA*	2.4 ± 0.1	B	2.5 ± 0.4	A	2.7 ± 0.05	A

Appendix C. Additional congo red binding phenotypes and pellicle assays of *P. putida* strains

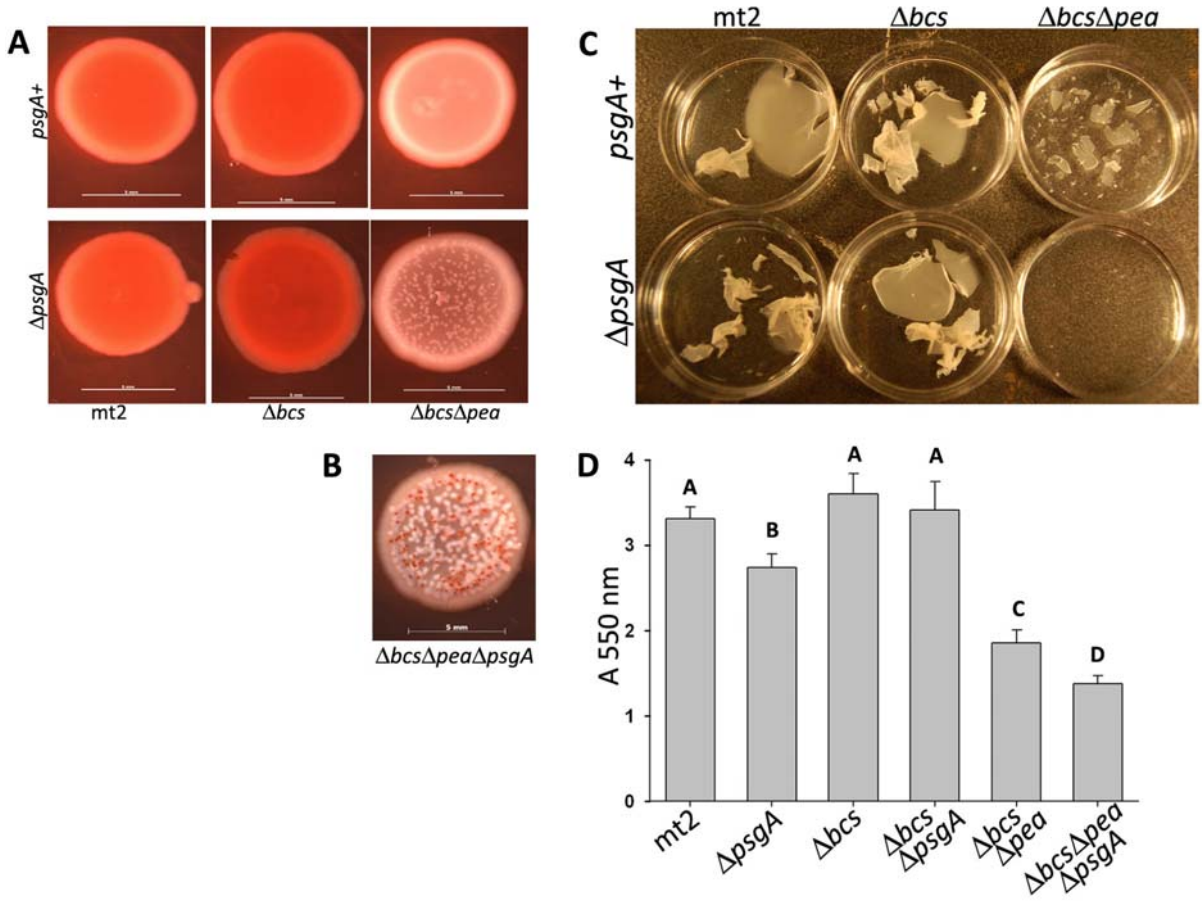

Figure C. Biofilm formation properties of *P. putida* and purli-deficient strains expressing pWspR19. Congo red binding phenotype of colonies on LB agar containing coomassie blue and congo red after 48 hours (A) and after 4 days (B). (C) Stability of pellicles formed in static cultures 24 h after inoculation in TYE broth amended with 0.3M NaCl. Pellicles were formed in a 6-well microtiter plate and then transferred using a wooden applicator stick to petri dishes filled with saline solution. (D) 24-hour microtiter plate biofilm assays.

Appendix D. Comparison of AlgU promoter regions amongst select Pseudomonads strains.

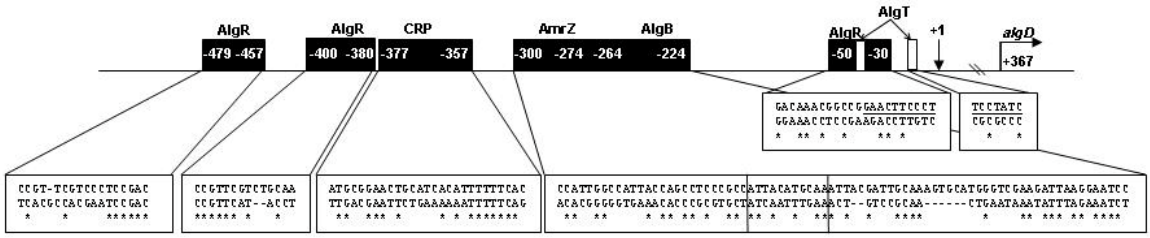

Figure D1. Schematic illustration and CLUSTALW alignment between *P. aeruginosa* PAO1 (top) and *P. putida* mt2 (bottom) *algD* promoter regions. Numbers inside black boxes represent the DNA binding sites relative to the PAO1 transcriptional start site for various regulatory proteins involved in activation of the *alg*D operon. The AmrZ and AlgB DNA binding sites overlap by 11 base pairs. Previously published: Li, X., Nielsen, L., Nolan, C. and Halverson, L. J. (2010), Transient alginate gene expression by *Pseudomonas putida*biofilm residents under water-limiting conditions reflects adaptation to the local environment. Environmental Microbiology, 12: 1578–1590.

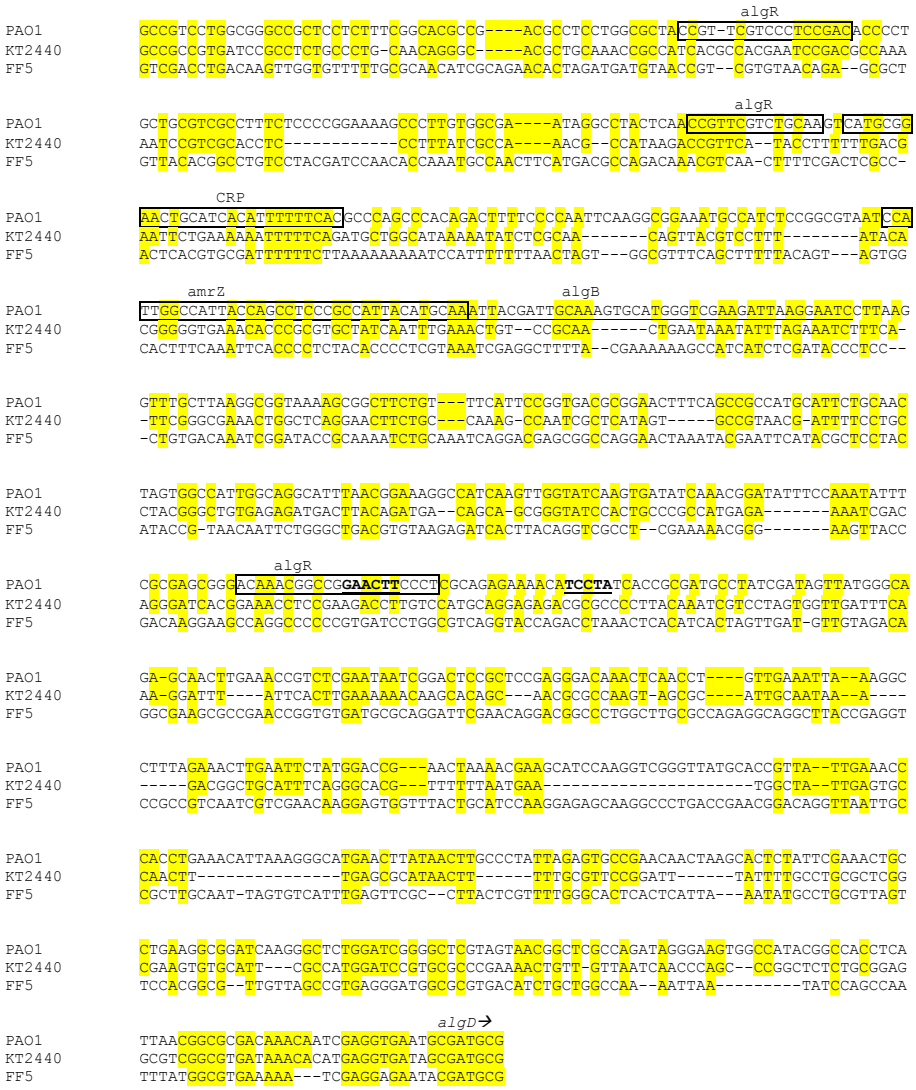

Figure D2. Sequence alignment of the *algD* promoter region of *P. aeruginosa* PAO1 (PAO1), *P. putida* KT2440 (KT2440), and *P. syringae* FF5 (FF5). AlgR, CRP, and AmrZ binding sites are boxed, the AlgB binding site is underlined, and the sigma factor AlgT recognition site is bold and underlined. The *algD* translational start site is indicated by *algD*→. Identical bases in the alignment between PAO1, KT2440, and FF5 are shaded. Gaps are indicated by dashes. The pairwise alignment between PAO1 and KT2440 was generated using ClustalW with a gap open penalty of 15.0 and gap extension of 6.66 using the IUB matrix. FF5 sequence was aligned to the PAO1 and KT2440 sequence using ClustalWProf using the same gap penalties listed above. The sequences selected for alignment were based on the promoter sequence used to construct our alginate reporter systems. Based on BlastP analysis at the Psuedomonas.com website, *P. putida* has homologs to the PAO1 σ^{22} (PP1427; 92% similarity), AlgR (PP0185; 88% similarity), AmrZ (PP4470; 93%similarity), AlgB (PP0133; 90% similarity), and CRP (PP0424; 85% similarity) transcriptional factors.

Appendix E. List of *P. putida* gene expression values induced significantly following a 15-minute solute or matric shock relative to water-replete conditions.

Table E. Induced genes

PP #	Gene	Gene Function	p-	q-	Solute	Matric
0085		conserved hypothetical protein	0.00	0.04	12.4	20.9
0086		conserved hypothetical protein	0.01	0.06	1.3[a]	1.9
0087		conserved hypothetical protein	0.01	0.07	1.6	2.5
0089	osmC	Osmotically inducible protein C	0.00	0.05	2.1	5.0
0090		conserved hypothetical protein	0.00	0.04	1.9	3.9
0115	katE	Catalase (EC 1.11.1.6)	0.00	0.05	1.3	2.4
0121	thrB	Homoserine kinase (EC 2.7.1.39)	0.03	0.11	1.1	1.5
0135		Probable transmembrane protein	0.00	0.01	21.6	14.2
0136		Inhibitor of vertebrate lysozyme precursor	0.00	0.02	16.3	12.8
0150		conserved hypothetical protein	0.04	0.11	1.3	2.9
0181		DNA-binding protein inhibitor Id-2-related	0.03	0.11	1.0	3.4
0185	pprA	Positive alginate biosynthesis regulatory	0.01	0.07	1.9	3.1
0186	hemC	Porphobilinogen deaminase (EC 2.5.1.61)	0.03	0.11	1.1	1.5
0188		uroporphyrinogen III methylase	0.04	0.11	1.4	2.1
0191	pfrA	Transcriptional regulatory protein algQ	0.05	0.13	0.9	1.7
0194	algP	Transcriptional regulatory protein algP	0.01	0.07	1.8	2.8
0201		conserved hypothetical protein	0.05	0.12	1.3	1.5
0213	gabD	Succinate-semialdehyde dehydrogenase	0.03	0.11	2.6	2.3
0214	gabT	4-aminobutyrate aminotransferase (EC	0.01	0.07	3.0	2.7
0216		Sensory box/GGDEF family protein	0.00	0.05	1.6	3.1
0224		Acyl-CoA dehydrogenase family protein	0.03	0.10	1.0	1.8
0235	lsfA	Peroxiredoxin	0.01	0.06	2.0	7.1
0236	ssuE	FMN reductase (EC 1.5.1.29)	0.05	0.12	1.7	4.9
0238	ssuD	Alkanesulfonate monooxygenase (EC 1.1.-.-)	0.03	0.10	1.2	3.0
0240	ssuB	ABC transporter ATP-binding protein	0.02	0.09	1.2	2.4
0241	ssuF	Organosulfonate utilization protein SsuF	0.05	0.12	1.0	3.0
0243	gshA	Probable glutamate--cysteine ligase (EC	0.02	0.10	1.6	2.3
0246	ompR	Transcriptional regulatory protein ompR	0.01	0.06	1.5	2.7
0247	envZ	Osmolarity sensor protein envZ (EC 2.7.3.-)	0.00	0.04	1.3	1.8
0252	hslO	33 kDa chaperonin	0.00	0.06	1.5	2.8
0333		conserved hypothetical protein	0.00	0.03	1.5	3.7
0368		Acyl-CoA dehydrogenase (EC 1.3.99.3)	0.04	0.12	2.2	15.1
0370		Acyl-CoA dehydrogenase (EC 1.3.99.3)	0.00	0.04	1.0	1.6

[a] Shaded cells have gene expressional values under significance cut-off of 1.5-fold

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
0373		Stress induced hydrophobic peptide	0.00	0.05	2.4	4.8
0435		Cell wall endopeptidase, family M23/M37	0.01	0.07	2.1	3.1
0504	oprG	Outer membrane protein	0.01	0.08	0.5	4.6
0549		Lipoprotein, putative	0.05	0.12	1.2	1.5
0564		conserved hypothetical protein	0.03	0.10	1.2	1.8
0565		MutT/nudix family protein	0.03	0.11	1.3	1.9
0625	clpB	ClpB protein	0.00	0.05	1.5	5.4
0673		conserved hypothetical protein	0.03	0.11	2.6	2.1
0679		Orotate phosphoribosyltransferase (EC	0.00	0.05	2.2	3.0
0722	prsA	Ribose-phosphate pyrophosphokinase (EC	0.02	0.09	1.7	1.0
0737		putative lipoprotein	0.01	0.06	3.6	9.5
0770		PemI-like protein	0.04	0.11	1.4	1.8
0771		PemK	0.04	0.11	1.2	1.5
0773		Outer membrane protein	0.00	0.05	2.1	3.8
0797		conserved hypothetical protein	0.00	0.06	11.1	8.1
0817		Aspartate aminotransferase (EC 2.6.1.1)	0.03	0.11	2.3	1.2
0822		probable capsule anchoring protein ybbB	0.05	0.13	1.2	1.5
0837		conserved hypothetical protein	0.01	0.06	2.2	5.0
0840	cysE	Serine acetyltransferase (EC 2.3.1.30)	0.05	0.12	1.3	1.6
0846	hscA	Chaperone protein hscA homolog	0.03	0.10	1.5	1.1
0850		Radical SAM family enzyme	0.02	0.09	1.3	1.5
0859		Hydrolase	0.04	0.11	1.1	1.7
0868		Glycine betaine transport ATP-binding	0.00	0.04	14.2	17.3
0869		Glycine betaine transport system permease	0.00	0.05	12.4	14.4
0870		Glycine betaine-binding protein	0.00	0.03	27.1	29.0
0871		Glycine betaine transport system permease	0.00	0.02	16.8	21.1
0886		conserved hypothetical protein	0.00	0.03	4.2	9.0
0939		Nitrilase homolog 1	0.00	0.05	1.3	1.7
0940	tldD	TldD protein	0.02	0.08	1.3	1.9
0951	rpoX	Probable sigma(54) modulation protein	0.04	0.11	1.5	2.0
0960	ttg2C	Mce-related protein	0.03	0.10	2.3	1.8
0961	ttg2D	Toluene tolerance protein Ttg2D	0.00	0.05	1.9	2.0
0999	arcC	Carbamate kinase (EC 2.7.2.2)	0.02	0.10	1.3	5.3
1000	argI	Ornithine carbamoyltransferase, catabolic	0.01	0.08	1.2	10.1
1001	arcA	Arginine deiminase (EC 3.5.3.6)	0.01	0.07	1.4	10.4
1022	zwf-1	Glucose-6-phosphate 1-dehydrogenase (EC	0.00	0.05	1.3	1.8
1023	pgl	6-phosphogluconolactonase (EC 3.1.1.31)	0.05	0.12	1.5	2.0
1024	eda	2-dehydro-3-deoxyphosphogluconate	0.04	0.11	1.1	1.6
1025	leuA	2-isopropylmalate synthase (EC 2.3.3.13)	0.00	0.05	0.9	1.6
1131		Outer membrane lipoprotein slyB precursor	0.02	0.08	2.2	1.9
1149		hypothetical protein	0.04	0.11	0.8	3.9

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
1185	oprH	outer membrane protein H1	0.00	0.04	38.6	1.7
1186	phoP	Transcriptional regulatory protein phoP	0.00	0.02	17.1	1.0
1187	phoQ	Sensor protein phoQ (EC 2.7.3.-)	0.00	0.03	2.3	0.9
1209		Cold shock protein	0.05	0.13	1.2	1.6
1210		Non-specific DNA-binding protein Dps / Iron-	0.02	0.08	1.2	4.1
1211		conserved hypothetical protein	0.05	0.13	1.2	2.3
1318	petB	Cytochrome b (EC 1.10.2.2)	0.01	0.07	0.6	1.8
1321	sspB	Stringent starvation protein B	0.03	0.10	1.4	1.8
1345	secA	Protein export cytoplasm protein (SecA,	0.03	0.10	1.6	2.6
1353		Mechanosensitive ion channel	0.01	0.08	3.8	3.4
1357		conserved hypothetical protein	0.00	0.05	2.1	3.7
1359	fxsA	FxsA protein	0.03	0.11	1.2	3.0
1360	groES	10 kDa chaperonin	0.01	0.07	6.0	10.3
1361	groEL	60 kDa chaperonin	0.01	0.07	6.6	13.1
1370		Glycosyl transferase, group 1 family protein	0.01	0.07	1.3	2.2
1384	ttgC	Outer membrane protein	0.02	0.09	1.6	1.6
1385	ttgB	RND multidrug efflux transporter	0.02	0.08	1.7	1.6
1386	ttgA	Periplasmic linker protein	0.04	0.12	2.1	1.9
1427	algU	RNA polymerase sigma-H factor	0.00	0.04	8.5	14.3
1428	mucA	Sigma factor algU negative regulatory	0.00	0.04	7.9	12.9
1429	algN	Sigma factor algU regulatory protein mucB	0.00	0.04	4.6	7.1
1430	algY	Protease Do (EC 3.4.21.-)	0.00	0.03	1.9	2.7
1443	lon-1	ATP-dependent protease La (EC 3.4.21.53)	0.02	0.08	1.5	4.1
1478		NADH-dependent flavin oxidoreductase	0.05	0.13	1.4	1.7
1502		Outer membrane porin F	0.00	0.05	5.0	12.9
1503		conserved hypothetical protein	0.00	0.04	15.3	31.0
1504		conserved hypothetical protein	0.01	0.06	1.6	4.2
1553		conserved hypothetical protein	0.02	0.08	1.1	1.7
1586		killer protein, putative	0.04	0.11	1.5	1.7
1616		Alcohol dehydrogenase class III (EC 1.1.1.1)	0.01	0.06	2.8	5.9
1617		hypothetical protein	0.00	0.04	1.4	2.6
1629	recA	RecA protein	0.03	0.10	1.8	2.3
1633		Putative secreted protein	0.01	0.06	2.8	4.8
1654	cysM	Cysteine synthase (EC 2.5.1.47)	0.02	0.10	1.6	1.8
1663		conserved hypothetical protein	0.03	0.11	1.3	1.8
1689		Long-chain fatty acid transport protein	0.04	0.11	2.8	2.4
1691		conserved domain protein	0.00	0.05	5.3	8.7
1714	fklB-2	Peptidyl-prolyl cis-trans isomerase (EC	0.02	0.09	1.9	4.2
1719	prc	Tail-specific protease (EC 3.4.21.-)	0.00	0.05	1.4	2.6
1732	rluA	Cell division topological specificity factor	0.02	0.10	1.6	1.8
1744		conserved hypothetical protein	0.00	0.05	2.0	6.2

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
1747		conserved hypothetical protein	0.00	0.06	2.9	4.3
1748		Deblocking aminopeptidase (EC 3.4.11.-)	0.00	0.04	5.5	11.6
1749		Cyanophycin synthetase (EC 6.-.-.-)	0.00	0.03	5.3	10.6
1750	asnB	Asparagine synthetase [glutamine-	0.00	0.04	6.2	16.3
1755	fumC-	Fumarate hydratase (EC 4.2.1.2)	0.00	0.05	1.6	1.8
1756		conserved hypothetical protein	0.00	0.05	4.1	5.5
1757	bolA	BolA protein	0.00	0.04	3.7	6.1
1763		Short chain dehydrogenase	0.04	0.11	1.3	1.5
1773	ihfB	Integration host factor beta-subunit	0.01	0.07	1.6	2.7
1797		Alkaline protease secretion protein aprE	0.04	0.11	1.4	1.5
1801	wbpY	Mannosyltransferase (EC 2.4.1.-)	0.04	0.11	1.3	1.5
1814		conserved hypothetical protein	0.00	0.05	1.4	2.2
1833		conserved hypothetical protein	0.01	0.06	9.0	8.1
1834		ankyrin domain protein	0.00	0.05	1.5	2.6
1840		conserved hypothetical protein	0.01	0.07	1.2	1.6
1912	plsX	Fatty acid/phospholipid synthesis protein	0.00	0.06	2.3	1.4
1918		conserved hypothetical protein TIGR00247	0.01	0.07	1.3	1.6
1961		identified by match to TIGR protein family	0.05	0.12	2.5	2.0
1982	ibpA	16 kDa heat shock protein B	0.01	0.08	3.8	10.3
2036		Dihydrodipicolinate synthase (EC 4.2.1.52)	0.00	0.05	1.2	3.3
2037		L-fuculose phosphate aldolase (EC 4.1.2.17)	0.01	0.07	1.0	3.4
2052		Phosphoglycolate phosphatase (EC 3.1.3.18)	0.00	0.04	11.1	10.1
2059		conserved hypothetical protein	0.00	0.05	1.8	2.6
2088	sigX	ECF sigma factor SigX (RNA polymerase	0.01	0.06	5.2	6.7
2089	oprF	OprF	0.02	0.08	1.2	1.8
2103		conserved hypothetical protein	0.00	0.04	3.1	5.2
2104		Probable transmembrane protein	0.00	0.04	2.9	6.0
2105		Probable transmembrane protein	0.00	0.02	15.1	27.1
2112	acnA	Aconitate hydratase (EC 4.2.1.3)	0.01	0.06	1.6	2.4
2121		Lipoprotein, putative	0.02	0.08	2.7	5.6
2132		Universal stress protein family	0.01	0.07	1.5	2.8
2136	fadB	Fatty oxidation complex alpha subunit	0.01	0.06	2.0	5.6
2137	fadA	3-ketoacyl-CoA thiolase (EC 2.3.1.16)	0.01	0.07	2.0	5.1
2144		Transcriptional regulator, TetR family	0.00	0.05	1.1	2.4
2154	lolC	Lipoprotein releasing system	0.01	0.06	1.2	1.7
2155	lolD	Lipoprotein releasing system ATP-binding	0.01	0.08	1.0	1.5
2161		conserved hypothetical protein	0.00	0.05	4.6	10.0
2168	tal	Transaldolase (EC 2.2.1.2)	0.01	0.07	1.2	1.9
2172		putative glycine-rich protein	0.03	0.11	1.8	2.4
2187		Universal stress protein family	0.03	0.10	1.1	1.7
2189		Probable transmembrane protein	0.02	0.09	1.5	1.7

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
2198		Glucose dehydrogenase B	0.00	0.06	1.2	1.5
2210		CbbR	0.03	0.10	1.1	1.5
2302	lon-2	ATP-dependent protease La (EC 3.4.21.53)	0.00	0.04	1.9	5.5
2303	hupB	DNA-binding protein HU-beta	0.03	0.10	1.1	1.5
2310		Methyl-accepting chemotaxis protein	0.05	0.12	0.9	1.5
2330		conserved hypothetical protein	0.04	0.11	1.5	1.8
2343		hypothetical protein	0.02	0.10	1.2	1.8
2356		Cyanobacterial phytochrome A (EC 2.7.3.-)	0.03	0.10	1.2	1.8
2430		Transcriptional regulator, AraC family	0.03	0.10	1.3	1.6
2448		Oxidoreductase (EC 1.1.1.-)	0.00	0.03	12.4	12.6
2462		Lipoprotein, putative	0.01	0.06	2.4	4.0
2474		glutathione S-transferase family protein	0.04	0.11	1.7	3.0
2475		Transcriptional regulator, TetR family	0.01	0.06	1.5	4.4
2476		Quinone oxidoreductase (EC 1.6.5.5)	0.01	0.07	1.1	2.2
2477		isoquinoline 1-oxidoreductase, alpha subunit	0.02	0.09	1.1	2.0
2478		Probable transmembrane isoquinoline 1-	0.03	0.11	1.3	2.0
2479		Cytochrome c	0.00	0.04	1.3	1.6
2488		Succinate-semialdehyde dehydrogenase	0.05	0.12	1.1	1.8
2489		NADH:flavin oxidoreductases, Old Yellow	0.03	0.11	1.2	2.5
2490		Oxygen-insensitive NADPH nitroreductase	0.02	0.09	1.1	2.1
2491		ThiJ/PfpI family protein	0.00	0.04	1.3	6.3
2492		NADH-dependent butanol dehydrogenase B	0.01	0.06	1.6	9.3
2499		Transcriptional regulator, CopG family	0.04	0.11	2.3	2.7
2500		conserved hypothetical protein	0.02	0.09	1.6	1.6
2581		hypothetical protein	0.04	0.11	1.3	2.1
2648		Universal stress protein family	0.04	0.12	0.9	2.3
2706		hypothetical protein	0.01	0.06	1.1	1.5
2725	pfpI	Protease I (EC 3.2.-.-)	0.00	0.02	1.2	1.6
2827		Quinone oxidoreductase	0.02	0.10	1.1	1.5
2852		sulfatase domain protein, putative	0.00	0.05	1.2	1.5
2874		hypothetical protein	0.01	0.06	1.2	11.3
2901		Aculeacin A acylase precursor (EC 3.5.1.-)	0.02	0.09	1.2	1.5
2918		Trehalose synthase	0.00	0.05	1.2	1.8
2947		hypothetical protein	0.01	0.06	2.7	4.5
2951		Transcriptional regulator, TetR family	0.03	0.10	1.5	1.8
2987		Transcriptional regulator, MarR family	0.02	0.10	1.3	1.5
3033		cI	0.03	0.10	2.9	5.2
3096		SciB protein	0.02	0.09	2.2	3.5
3097		ImpG	0.00	0.04	4.8	6.2
3098		ImpF	0.00	0.04	6.1	8.5
3099		SciI protein	0.00	0.05	6.8	8.9

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
3100		ImpB	0.00	0.03	21.5	29.8
3104		hypothetical protein	0.01	0.07	1.9	3.0
3105		hypothetical protein	0.03	0.11	1.4	1.8
3106		VgrG protein	0.03	0.10	2.2	2.2
3109		hypothetical protein	0.04	0.11	1.9	1.8
3111		hypothetical protein	0.04	0.11	1.3	1.6
3241		putative exported protein	0.00	0.04	1.8	3.4
3266		conserved hypothetical protein	0.01	0.07	1.3	1.9
3309		Oxidoreductase	0.05	0.12	1.2	1.6
3324		Regulatory protein	0.01	0.06	1.1	1.5
3358	ech	Enoyl-CoA hydratase/aldolase	0.00	0.03	1.2	1.5
3376	kguD	2-ketogluconate reductase (EC 1.1.1.215)	0.05	0.12	1.4	1.9
3380	ptxS	Kdg operon repressor	0.02	0.08	1.2	1.5
3383		gluconate 2-dehydrogenase (FAD) (EC	0.03	0.11	1.1	1.8
3401		hypothetical protein	0.01	0.08	1.4	1.7
3437		CBS domain protein	0.00	0.02	1.1	3.7
3476		Probable general secretion pathway GSPG-	0.01	0.07	1.2	1.5
3528		ABC transporter aliphatic-sulfonate-binding	0.02	0.10	1.1	1.6
3529		Dibenzothiophene desulfurization enzyme A	0.05	0.13	1.1	1.8
3541		Mg(2+) transport ATPase protein C	0.01	0.07	1.4	1.9
3563		probable transmembrane protein	0.00	0.04	1.4	2.1
3598		Putative glutamine amidotransferase	0.00	0.04	4.4	5.0
3613		L-sorbosone dehydrogenase	0.01	0.06	1.6	2.6
3631		conserved hypothetical protein	0.01	0.08	2.3	3.2
3704		hypothetical protein	0.00	0.04	3.3	3.5
3707		hypothetical protein	0.05	0.13	1.1	2.0
3743		conserved hypothetical protein	0.04	0.12	1.5	3.0
3757		Response regulator	0.00	0.04	3.4	5.7
3758		Sensor histidine kinase/response regulator	0.01	0.07	2.8	3.2
3759		Protein-glutamate methylesterase (EC	0.00	0.04	2.6	2.9
3760		Chemotaxis protein methyltransferase (EC	0.00	0.04	3.1	3.1
3761		Two-component hybrid sensor and regulator	0.03	0.10	2.4	3.4
3762		Histidine kinase/response regulator hybrid	0.01	0.06	1.8	6.0
3765		hypothetical protein	0.01	0.07	2.2	5.0
3768	aroE	Shikimate 5-dehydrogenase (EC 1.1.1.25)	0.02	0.08	1.4	1.9
3822		Cytochrome c family protein	0.00	0.02	1.0	1.6
3824		hypothetical protein	0.01	0.06	1.6	2.9
3832	csrA-	Carbon storage regulator homolog	0.00	0.04	4.3	7.6
3833		Two-component response regulator	0.04	0.11	1.1	1.5
3839	adhA	Alcohol dehydrogenase, propanol-preferring	0.02	0.10	1.0	4.3
3852		BNR domain protein	0.00	0.05	1.1	1.6

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
3923		phosphoglycerate mutase-related protein	0.02	0.09	1.1	1.9
3924		SCP-2 sterol transfer family protein	0.00	0.04	3.0	5.4
3928		conserved hypothetical protein	0.00	0.05	1.7	3.3
3929		hypothetical protein	0.01	0.07	4.0	8.2
3957		High-affinity choline transport protein	0.01	0.07	1.8	2.3
3958		hypothetical protein	0.00	0.05	1.4	2.3
3963		conserved domain protein	0.04	0.11	1.3	1.9
4000	serS	Seryl-tRNA synthetase (EC 6.1.1.11)	0.04	0.11	1.0	1.5
4004	ftsK	Cell division protein ftsK	0.01	0.07	1.4	2.1
4008	clpA	ATP-dependent clp protease ATP-binding	0.02	0.08	1.5	2.6
4009	clpS	Protein yljA	0.02	0.09	1.8	3.5
4032		Outer membrane lipoprotein Blc	0.01	0.06	1.5	3.0
4040		Glyoxalase family protein	0.04	0.11	1.3	1.7
4041		Glucoamylase (EC 3.2.1.3)	0.01	0.07	1.3	2.0
4042	zwf-2	Glucose-6-phosphate 1-dehydrogenase (EC	0.01	0.06	1.8	3.2
4043	gnd	6-phosphogluconate dehydrogenase (EC	0.00	0.04	1.8	3.3
4050	glgA	Glycogen synthase (EC 2.4.1.21)	0.00	0.04	2.8	4.7
4051		Malto-oligosyltrehalose trehalohydrolase (EC	0.00	0.06	2.0	2.3
4052	malQ	4-alpha-glucanotransferase (EC 2.4.1.25)	0.01	0.07	1.5	1.6
4054		conserved hypothetical protein	0.02	0.08	1.5	2.3
4055	glgX	Glycogen debranching enzyme	0.00	0.02	1.5	1.7
4056		Endonuclease/Exonuclease/phosphatase	0.00	0.05	1.2	1.5
4059		Trehalose synthase	0.04	0.11	1.4	1.6
4060		Alpha-amylase	0.02	0.08	1.5	3.1
4069		hypothetical protein	0.03	0.11	1.8	2.8
4072		conserved hypothetical protein	0.00	0.05	1.3	1.7
4099	gacA	Response regulator gacA	0.01	0.07	1.2	1.9
4139		hypothetical protein	0.01	0.07	3.2	8.1
4167	sixA	Phosphohistidine phosphatase sixA (EC	0.01	0.07	1.2	1.6
4170		hypothetical protein	0.01	0.06	1.2	1.8
4176		5-oxo-L-prolinase, putative	0.00	0.04	1.1	1.6
4178		Dienelactone hydrolase family protein	0.00	0.05	1.9	4.9
4179	htpG	Chaperone protein htpG	0.01	0.06	4.8	14.4
4182		identified by match to PFAM protein family	0.01	0.06	1.1	1.7
4201		Electron transfer flavoprotein alpha-subunit	0.00	0.05	1.6	3.4
4202		Electron transfer flavoprotein beta-subunit	0.00	0.02	1.6	2.4
4203		Electron transfer flavoprotein-ubiquinone	0.03	0.11	1.9	4.5
4205		conserved hypothetical protein	0.03	0.10	1.4	1.5
4220		non-ribosomal peptide synthetase domain	0.04	0.11	1.3	1.8
4260		conserved hypothetical protein	0.04	0.11	1.1	1.7
4265	anr	Transcriptional activator protein fnrA	0.04	0.11	1.3	1.6

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
4319		Lipoprotein, putative	0.04	0.11	1.3	1.8
4362		Hpt domain protein	0.00	0.05	1.2	1.5
4470	algZ	Alginate biosynthesis transcriptional	0.03	0.10	2.7	3.4
4498		Dihydrofolate reductase (EC 1.5.1.3)	0.03	0.11	1.1	1.5
4528		conserved hypothetical protein	0.00	0.05	1.5	2.9
4541	iA	Peptidyl-prolyl cis-trans isomerase A	0.04	0.12	2.4	3.1
4549	fadD	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	0.02	0.08	1.4	1.6
4560		Ribonuclease BN (EC 3.1.-.-)	0.01	0.07	1.4	2.8
4561		conserved hypothetical protein	0.00	0.03	5.7	10.4
4573		ATPase, AAA family	0.05	0.12	1.3	1.9
4581		Putative amidotransferase	0.03	0.11	1.3	1.6
4614		hypothetical protein	0.03	0.10	3.0	4.2
4645	mscL	Large-conductance mechanosensitive	0.02	0.10	1.5	3.1
4690		Iron-sulfur cluster-binding protein, Rieske	0.03	0.10	1.3	1.5
4693		DnaK suressor protein	0.03	0.11	1.2	1.5
4704		putative exported protein	0.02	0.10	1.6	1.7
4707		OsmY-related protein	0.00	0.02	54.1	52.5
4708	pnp	Polyribonucleotide nucleotidyltransferase	0.04	0.11	1.7	1.7
4716	glmM	Protein mrsA homolog	0.03	0.11	1.3	1.5
4717	folP	Dihydropteroate synthase (EC 2.5.1.15)	0.00	0.04	1.3	1.7
4718	ftsH	Cell division protein ftsH (EC 3.4.24.-)	0.00	0.05	1.2	1.9
4725	dapB	Dihydrodipicolinate reductase (EC 1.3.1.26)	0.01	0.07	1.5	2.7
4726	dnaJ	Chaperone protein dnaJ	0.03	0.11	1.8	3.5
4727	dnaK	Chaperone protein dnaK	0.00	0.04	5.2	15.2
4728	grpE	GrpE protein	0.00	0.04	8.1	20.7
4736	lldD	L-lactate dehydrogenase (EC 1.1.2.3)	0.04	0.11	1.0	3.6
4738		conserved hypothetical protein	0.00	0.05	2.4	5.5
4770		conserved hypothetical protein	0.01	0.06	1.2	2.2
4773		conserved hypothetical protein	0.01	0.07	1.4	3.2
4817		MaoC domain protein	0.01	0.08	1.1	2.1
4847		conserved hypothetical protein	0.01	0.06	1.1	1.8
4848		Curved DNA-binding protein	0.01	0.08	1.1	1.7
4855	osmE	Osmotically-inducible lipoprotein OsmE	0.00	0.03	18.7	35.0
4856		Bacterioferritin	0.00	0.03	7.0	15.1
4870		Azurin	0.01	0.06	0.5	2.8
4891	hflC	HflC protein (EC 3.4.-.-)	0.03	0.11	1.7	2.5
4892	hflK	HflK protein	0.03	0.10	2.2	3.4
4893	hflX	GTP-binding protein hflX	0.01	0.06	1.9	3.2
4894	hfq	Hfq protein	0.01	0.07	1.9	3.7
4895	miaA	tRNA delta(2)-isopentenylpyrophosphate	0.03	0.11	1.6	1.1
4919		ADP-ribose pyrophosphatase (EC 3.6.1.13)	0.04	0.11	0.9	1.5

Table E. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
4978		conserved hypothetical protein	0.05	0.12	1.2	1.7
5000	hslV	ATP-dependent protease hslV (EC 3.4.25.-)	0.00	0.05	3.9	11.3
5001	hslU	ATP-dependent hsl protease ATP-binding	0.00	0.04	2.8	10.8
5007		PhaF protein	0.03	0.10	1.1	1.9
5008		PhaI protein	0.03	0.11	1.0	1.9
5026	mdoG	Periplasmic glucans biosynthesis protein	0.03	0.10	2.0	3.9
5037		Outer membrane lipoprotein Blc	0.00	0.04	1.1	1.6
5038		Lipoprotein, putative	0.00	0.05	1.2	2.0
5041	glgP	Glycogen phosphorylase (EC 2.4.1.1)	0.01	0.07	1.4	1.8
5064	betA	Choline dehydrogenase (EC 1.1.99.1)	0.02	0.08	1.6	1.3
5108	rpoH	RNA polymerase sigma-32 factor	0.00	0.04	1.9	6.1
5156		conserved hypothetical protein	0.00	0.05	1.6	2.1
5166		Transcriptional regulatory protein	0.05	0.12	1.0	1.7
5167		conserved hypothetical protein	0.03	0.11	1.6	3.2
5171	cysP	Sulfate-binding protein	0.03	0.10	1.7	3.0
5172		conserved hypothetical protein	0.02	0.08	2.0	5.8
5184		Glutamine synthetase (EC 6.3.1.2)	0.01	0.06	3.4	4.7
5206		periplasmic component of efflux system	0.02	0.10	1.1	2.6
5220	elbB	Sigma cross-reacting protein 27A	0.02	0.09	1.2	1.6
5238		putative cytoplasmic protein	0.00	0.04	1.8	3.7
5267		Cytochrome c5	0.01	0.06	0.8	1.6
5278		Aldehyde dehydrogenase (EC 1.2.1.3)	0.00	0.04	15.7	18.1
5279		protein	0.00	0.04	12.8	14.2
5288		Phosphomannomutase (EC 5.4.2.8)	0.00	0.05	2.3	2.9
5289	argB	Acetylglutamate kinase (EC 2.7.2.8)	0.05	0.13	1.4	1.9
5297		Amino acid permease	0.02	0.09	2.0	0.9
5299		Glutamine synthetase (EC 6.3.1.2)	0.01	0.07	4.1	2.5
5302	spoT	Guanosine-3',5'-bis(Diphosphate) 3'-	0.04	0.11	1.7	1.7
5303		Translation initiation inhibitor	0.02	0.09	1.5	1.7
5304		Lipoprotein, putative	0.03	0.10	1.6	1.7
5333		Lipoprotein, putative	0.00	0.04	1.6	2.5
5334		putative membrane protein	0.00	0.04	1.6	4.4
5353		putative membrane protein	0.00	0.03	1.7	3.7
5363		conserved hypothetical protein	0.00	0.05	1.3	2.3
5389		identified by match to PFAM protein family	0.00	0.05	0.9	3.3
5391		hypothetical protein	0.01	0.06	0.7	7.0
5392		Surface antigen gene	0.00	0.05	0.9	6.1
5409	glmS	Glucosamine--fructose-6-phosphate	0.01	0.07	1.2	1.8

Appendix F. List of *P. putida* gene expression values repressed significantly following a 15-minute solute or matric shock relative to water-replete conditions.

Table F. Repressed Genes

	Gene	Gene Function	p-	q-	Solute	Matric
0024		Integral membrane protein	0.00	0.04	1.4[a]	1.5
0033		Dolichyl-phosphate-mannose-protein mannosyltransferase family protein	0.00	0.03	1.7	2.0
0034		Bactoprenol glucosyl transferase (EC 2.4.1.-)	0.01	0.06	1.3	1.5
0035		GtrA family protein	0.01	0.06	1.6	2.1
0039		hypothetical protein	0.00	0.05	1.9	2.3
0048		hypothetical protein	0.02	0.09	1.3	1.5
0078		hypothetical protein	0.01	0.07	1.3	1.5
0093		PUTATIVE ZINC PROTEASE PROTEIN	0.01	0.07	1.4	1.6
0146		Integral membrane protein	0.03	0.10	1.6	1.8
0276		Transcriptional regulator, Cro/CI family	0.00	0.02	1.3	1.5
0278		hypothetical protein	0.02	0.09	1.5	1.8
0299		conserved hypothetical protein	0.03	0.10	1.5	1.5
0331		YD repeat protein	0.02	0.09	1.5	1.7
0353		Exonuclease	0.01	0.06	1.6	2.0
0405		7.5 kDa CHLOROSOME PROTEIN	0.01	0.06	1.2	1.7
0443	rplK	50S ribosomal protein L11	0.01	0.06	1.2	1.9
0444	rplA	50S ribosomal protein L1	0.03	0.10	1.2	1.8
0445	rplJ	50S ribosomal protein L10	0.00	0.05	1.4	1.7
0453	rpsJ	Ribosomal protein S10	0.01	0.07	1.2	1.8
0454	rplC	50S ribosomal protein L3	0.02	0.10	1.3	1.8
0479	rpoA	DNA-directed RNA polymerase alpha chain (EC 2.7.7.6)	0.02	0.10	1.1	1.5
0525		Vitamin B12 receptor	0.00	0.04	1.4	1.7
0560	aroQ-1	3-dehydroquinate dehydratase 1 (EC 4.2.1.10)	0.03	0.10	1.1	2.6
0619		ABC transporter substrate-binding protein	0.01	0.06	1.2	1.5
0638		ISPpu15, transposase Orf1	0.01	0.06	2.3	6.7
0677		Lipoprotein, putative	0.03	0.10	1.2	1.6
0714		Transporter, PerM family	0.01	0.06	1.3	1.5
0726		probable sugar nucleotidyltransferase Cj1416c	0.01	0.08	1.2	1.5
0745	uraA	Uracil permease	0.03	0.10	1.4	1.7
0746	upp	Uracil phosphoribosyltransferase (EC 2.4.2.9)	0.01	0.07	1.6	2.3

[a] Shaded cells have gene expressional values below significance cut-off of 1.5-fold

Table F. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
0812	cyoA	Ubiquinol oxidase polypeptide II precursor (EC 1.10.3.-)	0.02	0.08	1.9	3.0
0814	cyoC	Cytochrome O ubiquinol oxidase subunit III (EC 1.10.3.-)	0.01	0.07	2.4	2.4
0862		Iron-uptake factor	0.01	0.06	1.3	1.5
0876		Transcriptional regulator, AraC family	0.02	0.08	1.3	1.5
0898		hypothetical protein	0.01	0.06	1.0	1.9
0985		Cold shock domain family protein	0.00	0.05	2.8	2.4
0988	gcvP-1	Glycine dehydrogenase [decarboxylating] (EC 1.4.4.2)	0.01	0.07	1.9	1.5
0989	gcvH	Glycine cleavage system H protein	0.00	0.05	4.9	5.7
1008		RNA polymerase sigma-70 family protein	0.01	0.07	1.4	1.6
1015		Sugar ABC transporter, periplasmic sugar-binding protein	0.03	0.10	1.5	2.9
1016		Permease of ABC sugar transporter	0.02	0.09	1.6	3.0
1017		Permease of ABC sugar transporter	0.03	0.10	1.3	2.8
1029		putative membrane protein	0.02	0.09	1.1	1.5
1033		sulfatase domain protein	0.02	0.08	1.3	1.6
1065		Membrane protein glpM	0.00	0.05	1.3	1.5
1068		Glutamate/aspartate transport ATP-binding protein gltL	0.01	0.07	1.4	2.3
1069		Glutamate/aspartate transport system permease protein gltK	0.01	0.06	1.4	2.4
1070		Glutamate/aspartate transport system permease protein gltJ	0.00	0.05	1.4	3.4
1071		Glutamate/aspartate periplasmic binding protein precursor	0.02	0.10	1.2	2.8
1091		conserved hypothetical protein	0.03	0.10	1.6	1.7
1099		Cold shock protein	0.00	0.05	1.8	3.1
1157		Acetolactate synthase, catabolic, putative	0.00	0.04	2.8	3.2
1188	dctA	C4-dicarboxylate transport protein 2	0.00	0.03	2.5	6.9
1223	oprL	Peptidoglycan-associated lipoprotein	0.01	0.07	2.3	2.3
1243		hypothetical protein	0.00	0.04	2.3	3.7
1245		17 kDa surface antigen precursor	0.01	0.07	2.1	3.6
1246		conserved domain protein	0.02	0.09	1.4	1.7
1280	algI	Membrane-bound O-acyltransferase	0.01	0.07	1.5	1.7
1318	petB	Cytochrome b (EC 1.10.2.2)	0.01	0.07	1.6	0.5
1363		Lipoprotein, putative	0.00	0.04	1.2	1.5
1612	eno	Enolase (EC 4.2.1.11)	0.00	0.05	1.0	1.5
1624		Group II intron-encoding maturase	0.03	0.10	1.5	2.7
1786		Glycosyl transferase, putative	0.02	0.09	2.0	2.7
1792		Glycosyltransferase (EC 2.4.1.-)	0.01	0.07	1.5	1.7

Table F. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
1916	fabF	3-oxoacyl-[acyl-carrier-protein] synthase (EC 2.3.1.41)	0.02	0.10	1.3	1.6
2095	pyrD	Dihydroorotate dehydrogenase (EC 1.3.3.1)	0.00	0.05	1.1	1.5
2114		ISPpu8, transposase	0.01	0.07	1.5	1.7
2134		ISPpu10, transposase	0.01	0.07	2.2	3.9
2218		ISPpu8, transposase	0.01	0.06	1.6	1.9
2221		hypothetical protein	0.01	0.07	1.3	1.5
2249		Chemotactic transducer	0.03	0.10	1.1	1.6
2254		Transcriptional regulator, GntR family	0.02	0.09	1.2	1.7
2522		ISPpu8, transposase	0.01	0.06	1.4	1.5
3009		hypothetical protein	0.01	0.06	1.2	1.6
3011		hypothetical protein	0.00	0.05	1.4	1.7
3172		Group II intron-encoding maturase	0.01	0.07	1.6	3.0
3305		Tellurium resistance protein terC	0.02	0.09	1.2	1.5
3365		Acetolactate synthase, catabolic, putative	0.01	0.08	3.1	4.0
3418		hypothetical protein	0.02	0.08	1.7	3.2
3499		ISPpu14, transposase Orf1	0.01	0.08	2.0	2.9
3511	ilvE	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	0.01	0.06	1.8	1.6
3595		Amino acid ABC transporter permease protein	0.02	0.09	1.1	1.6
3619		hypothetical protein	0.01	0.06	1.3	1.6
3820		Group II intron-encoding maturase	0.01	0.07	1.6	2.9
3838		hypothetical protein	0.00	0.05	1.6	1.9
3851		hypothetical protein	0.01	0.07	1.3	1.7
3966		ISPpu14, transposase Orf1	0.00	0.05	2.2	4.4
3979		ISPpu14, transposase Orf1	0.01	0.06	2.0	3.8
4024		ISPpu15, transposase Orf1	0.01	0.06	2.5	6.9
4062		hypothetical protein	0.00	0.04	1.3	1.5
4086		conserved domain protein	0.02	0.10	1.2	1.5
4087		hypothetical protein	0.01	0.07	1.3	3.0
4092		ISPpu15, transposase Orf1	0.01	0.06	2.3	6.7
4095		conserved domain protein	0.02	0.10	1.4	1.9
4174	fabA	3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (EC 4.2.1.60)	0.03	0.10	1.7	1.8
4183		conserved hypothetical protein	0.00	0.05	1.2	1.5
4192	sdhD	Succinate dehydrogenase hydrophobic membrane anchor protein	0.02	0.10	1.7	2.0
4193	sdhC	Succinate dehydrogenase cytochrome b-556 subunit	0.00	0.05	1.6	2.2
4293		conserved hypothetical protein	0.01	0.06	1.9	2.0
4308		Leucine-responsive regulatory protein	0.03	0.10	1.0	1.5

Table F. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
4318		ISPpu8, transposase	0.01	0.06	1.6	2.0
4331		conserved hypothetical protein	0.01	0.06	1.6	1.5
4354	fliQ	Flagellar biosynthetic protein fliQ	0.00	0.03	1.5	1.6
4364		Anti-sigma F factor antagonist, putative	0.00	0.06	1.2	1.6
4385	flgG	Flagellar basal-body rod protein flgG	0.01	0.08	1.8	1.9
4388	flgE	Flagellar hook protein flgE	0.02	0.10	1.9	2.1
4389	flgD	Basal-body rod modification protein flgD	0.02	0.08	1.3	1.7
4391	flgB	Flagellar basal-body rod protein flgB	0.01	0.07	1.5	1.4
4409		Site-specific recombinase, phage integrase family	0.00	0.05	1.4	1.6
4437		ISPpu14, transposase Orf1	0.01	0.06	2.1	3.6
4441		ISPpu14, transposase Orf1	0.01	0.07	2.1	3.2
4454		Dipeptide transport system permease protein dppC	0.02	0.10	1.3	1.6
4487	acsA	Acetyl-coenzyme A synthetase (EC 6.2.1.1)	0.02	0.08	1.4	1.6
4538		Acyl carrier protein phosphodiesterase (EC 3.1.4.14)	0.03	0.10	1.6	1.9
4543		conserved hypothetical protein	0.01	0.06	1.2	1.5
4566		Transporter, Drug/Metabolite Exporter family	0.01	0.06	1.5	1.8
4626		Murein hydrolase exporter	0.01	0.07	1.3	1.5
4746		ISPpu15, transposase Orf1	0.01	0.07	2.1	6.4
4821	fis	DNA-binding protein fis	0.02	0.10	1.0	1.7
4905		Chemotaxis motA protein	0.00	0.05	1.2	1.8
4960	fda	Fructose-bisphosphate aldolase (EC 4.1.2.13)	0.00	0.04	1.3	1.5
4967	metK	S-adenosylmethionine synthetase (EC 2.5.1.6)	0.02	0.08	2.0	2.1
5028	pip	Proline iminopeptidase (EC 3.4.11.5)	0.01	0.06	1.5	1.5
5031		Proline-specific permease	0.02	0.08	1.3	1.6
5043		conserved hypothetical protein	0.00	0.05	1.3	1.5
5046	glnA	Glutamine synthetase (EC 6.3.1.2)	0.00	0.05	5.3	4.7
5047	ntrB	Nitrogen regulation protein ntrB (EC 2.7.3.-)	0.01	0.08	1.7	1.8
5148		conserved hypothetical protein	0.01	0.06	1.6	2.0
5232		conserved hypothetical protein	0.00	0.02	3.4	3.9
5234		Nitrogen regulatory protein P-II	0.01	0.07	2.3	1.9
5244		Transcriptional regulator, AraC family	0.00	0.04	1.3	1.7
5248		Hydrolase (EC 1.14.-.-)	0.00	0.04	1.4	1.7
5255		Hydrolase (EC 1.14.-.-)	0.01	0.06	1.4	2.2
5275		Transcriptional regulatory protein	0.03	0.10	1.3	1.5
5290		ISPpu10, transposase	0.03	0.10	1.9	2.9
5343		Transcriptional regulator	0.01	0.07	1.2	1.7
5344		Acetyltransferase	0.01	0.08	1.3	1.6

Table F. (continued)

PP #	Gene	Gene Function	p-	q-	Solute	Matric
5356		Acyl-CoA hydrolase (EC 3.1.2.20)	0.01	0.07	1.2	1.5
5375		Glycine cleavage system transcriptional activator	0.02	0.08	1.4	1.9
5388		conserved hypothetical protein	0.02	0.09	1.0	1.5
5398		ISPpu14, transposase Orf1	0.01	0.06	2.2	3.1
5405		Transposon Tn7 transposition protein tnsB	0.01	0.07	1.3	1.6
5415	atpA	ATP synthase alpha chain (EC 3.6.3.14)	0.00	0.05	1.6	2.1
5416	atpH	ATP synthase delta chain (EC 3.6.3.14)	0.01	0.08	1.7	2.3
5418	atpE	ATP synthase C chain (EC 3.6.3.14)	0.01	0.06	1.7	1.9